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# Heteroplasmy in mammal mitochondrial deoxyribonucleic acid

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# Résumé

La nature a développé diverses stratégies afin d'assurer le commencement de la vie dans des conditions d'homoplasmie, c'est-à-dire des conditions telles que les cellules sont dotées du même ADN mitochondrial. Toutefois, des nouveaux haplotypes de l'acide désoxyribonucléique mitochondrial (ADNmt) peuvent apparaitre et croître de plusieurs façons tout au long de la durée d'une vie menant à l'hétéroplasmie. Par exemple, l'hétéroplasmie de l'ADNmt peut être créée artificiellement par des technologies reproductives assistées, ainsi que naturellement par le processus de vieillissement. De ce fait, la thèse de ce doctorat fut divisée en deux principaux objectifs. Le premier étant celui d'analyser les changements survenus dans l'hétéroplasmie de l'ADNmt produit par le transfert nucléaire des cellules somatiques (SCNT) lors du développement de l'embryon jusqu'au fœtus et aux tissus adultes de bovins clonés. En ce qui concerne le second objectif, il s'agit d'analyser les changements survenus dans l'hétéroplasmie de l'ADNmt causés par le vieillissement dans une cellule somatique adulte et dans des tissus germinaux durant l'ovogénèse, ainsi qu'au début de l'embryogenèse et dans la procédure de culture in vitro sur des souris.

Dans la première série d'expériences sur des bovins, des fibroblastes fœtaux transportant une mutation d'ADNmt (insertion de 66 pb) furent fusionnés avec des ovocytes receveurs transportant l'ADNmt du type sauvage. La présence d'ADNmt venant de la cellule donneuse a été analysée à différents stades de développement, soit sur des embryons âgés de 17 jours (n=17), des fœtus âgés de 40 jours (n=3), des fœtus âgés de 60 jours (n=3), un fœtus âgé de 240 jours et 3 clones post-nataux âgés de 18 à 24 mois.

Chaque individu s'est avéré être hétéroplasmique et 99 % (103/104) des échantillons de tissus analysés étaient également hétéroplasmiques. Cependant, l'ovaire venant du fœtus de 240 jours fut le seul à être homoplasmique pour l'ADNmt de l'ovocyte receveur. Dans la plupart des échantillons analysés (95,2 %, soit 99/104) la moyenne d'hétéroplasmie était de 1,46 %. Par contre, un fœtus âgé de 40 jours a présenté un niveau élevé d'hétéroplasmie (20,9 %), indiquant ainsi que des évènements rares d'augmentation de l'ADNmt des cellules donneuses peuvent survenir. Étant donné que la majorité des clones SCNT montrait de l'hétéroplasmie de l'ADNmt à des proportions comparables à celles des cellules donneuses au moment de la reconstruction de l'embryon, on a pu conclure que l'hétéroplasmie produite par des techniques de transfert nucléaire utilisant des cellules somatiques est due à une ségrégation neutre de l'ADNmt.

Dans la seconde série d'expériences sur des souris, des femelles de différents âges, c.à.d. jeunes (0 – 8 mois), moyennes (8 – 16 mois) et vieilles (16 – 24 mois), ont été synchronisées (gonadotrophines) et sacrifiées dans le but d'obtenir des ovocytes au stade de vésicule germinal, et des ovocytes au stade métaphase-II produits in vivo et in vitro. De plus, des embryons in vivo et in vitro au stade de deux-cellules et des embryons au stade de blastocystes ont été obtenus de femelles jeunes. Différents tissus somatiques, venant de femelles des trois stades d'âge ont été obtenus : cerveau, foie, muscle et du cumulus ovocytaire. De plus, l'effet du vieillissement a été mesuré selon la fertilité de la femelle. En effet, les effets sur l'hétéroplasmie du vieillissement, du stade de développement et de la culture in vitro ont été mesurés dans des ovocytes et dans des embryons. Les effets du vieillissement sur les mitochondries ont été mesurés par rapport au nombre total de copies

de l'ADNmt, au pourcentage des délétions communes et sur l'expression de trois gènes : Ndufs4, Mt-nd2 and Mt-nd4. Il a été possible d'observer que la fertilité des femelles dans la colonie de souris diminuait avec l'âge. En fait, le vieillissement affectait l'ADNmt dans les tissus somatiques, cependant il n'avait pas d'effet sur le cumulus, les ovocytes et les embryons. Le nombre de délétions de l'ADNmt augmentait pendant la reprise de la méiose et celui-ci diminuait au début du développement embryonnaire. La culture in vitro n'affectait pas la quantité d'ADNmt dans la plupart des tissus germinaux. Puisque nous n'avons pas trouvé d'effet de l'âge dans la majorité des paramètres mitochondriaux analysés dans les ovocytes et les embryons, il est suggéré que la délétion commune de l'ADNmt dans les tissus germinaux est davantage reliée au statut cellulaire de la production d'énergie qu'au processus de vieillissement.

Deux sources différentes de mutations de l'ADNmt produites dans les ovocytes normaux ou reconstitués ont produit différents résultats d'hétéroplasmie au début de l'embryogénèse. Chez les bovins, l'hétéroplasmie artificielle impliquant une petite insertion (66 pb) dans la région non codante (D-loop) de l'ADNmt a été vraisemblablement non nocive pour l'embryon, tolérant la persistance de l'ADNmt étranger pendant les différents stades du développement des clones. Chez les souris, l'hétéroplasmie naturelle produite par une grande délétion (4974 pb délétion commune) dans la région codante de l'ADNmt a été vraisemblablement nocive pour l'embryon et par conséquent éliminée pour assurer l'homoplasmie au début du développement embryonnaire.

**Mots clés** : Hétéroplasmie, transfert nucléaire des cellules somatiques (SCNT) bovine, ovogénèse de souris, début du développement embryonnaire chez la souris, vieillissement, culture in vitro, quantité totale d'ADNmt, délétion commune de l'ADNmt.

# Abstract

Nature has developed strategies to ensure the beginning of life in conditions of homoplasmy, i.e. cells harboring the same mitochondrial DNA (mtDNA). However, novel mtDNA haplotypes can arise by many means during life, leading to heteroplasmy. For instance, mtDNA heteroplasmy can originate artificially through assisted reproductive technologies and naturally by the process of aging. Therefore, this doctoral thesis was divided into two general objectives: Firstly, to analyze the changes in mtDNA heteroplasmy produced by somatic cell nuclear transfer (SCNT) during development from embryos, to fetuses and adult tissues, in cattle. Secondly, to analyze the changes in mtDNA heteroplasmy caused by aging in adult germinal and somatic tissues, during oogenesis and early embryogenesis, and in *in vitro* culture procedures in mice.

In the first series of experiments in cattle, fetal fibroblasts carrying an mtDNA mutation (insertion of 66 bp) were fused to host oocytes carrying wild type mtDNA. The presence of mtDNA from the donor cell was analyzed in 30 SCNT clones at different stages of development: 17-day-old embryos (n=17); 40-day-old fetuses (n=3); 60-day-old fetuses (n=3); one 240 day-old fetus; and 3 post-natal clones (18-24 months). Every individual clone proved to be heteroplasmic and 99% (103/104) of the analyzed tissue samples were heteroplasmic as well. Only the ovary coming from a 240 day old fetus was homoplasmic for the mtDNA of the recipient oocyte. In most (95.2%) of the analyzed tissue samples (99/104) the mean of heteroplasmy was 1.46%. In contrast, one 40-day-old fetus presented high levels of heteroplasmy (20.9%) indicating rare events of donor mtDNA increases. Since most SCNT clones showed heteroplasmy at proportions

comparable to the donor mtDNA at the moment of embryo reconstruction, we concluded that heteroplasmy produced by nuclear transfer techniques using somatic cells is due to the neutral segregation of the mtDNA.

In the second series of experiments, performed in mice, females of different ages, i.e. young (0-8 months), middle (8-16 months) and old (16-24 months), were synchronized (gonadotropins) and sacrificed to obtain germinal vesicle oocytes, metaphase-II oocytes in vivo and in vitro. Also, 2-cell and blastocyst stage embryos were obtained from young females in vivo and in vitro. Somatic tissues from females of the three age periods were obtained: brain, granulosa, liver and muscle and the effect of aging was measured on fertility. The effects of aging, stage of development and in vitro culture on the heteroplasmy were measured in oocytes and embryos. Also, the effects of aging were measured in somatic and germinal tissues on total copies of mtDNA, percentage of mtDNA common deletion and the expression of three genes: Ndufs4, Mt-nd2 and Mt-nd4. We observed that female fertility in the mouse colony decreases with age. Aging affected mtDNA in somatic tissues but no effect was observed in granulosa, oocytes and embryos. MtDNA deletions increased during the resumption of meiosis and decreased during early embryo development; and culture in vitro did not affect the mtDNA in most germinal tissues. Because we did not find effects of age in most mitochondrial parameters analyzed in oocytes and embryos, we suggest that mtDNA common deletion in germinal tissues is more related with the cellular status of energy production than with the process of aging.

Two different sources of mutations in the mtDNA generated in normal or reconstructed oocytes produced different heteroplasmy outcomes at the beginning of

embryogenesis. In cattle, artificial heteroplasmy involving a small insertion (66 bp) in the non coding region (D-loop) of the mitochondrial DNA was apparently not harmful to the embryo, allowing persistence of the foreign mtDNA during the different stages of clonal development. In mice, the natural heteroplasmy of a large deletion (4974 bp, common deletion) in the coding region of the mtDNA was apparently harmful to the embryo and, therefore, may have been eliminated to ensure homoplasmy at the beginning of embryonic development.

**Keywords** : Heteroplasmy, bovine SCNT, neutral segregation of mtDNA, mouse oogenesis, mouse early embryo development, aging, culture in vitro, total mtDNA copies, mtDNA common deletion.

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*To my family*

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# List of abbreviations

| | |
|---|---|
| Ad | Adult |
| AE | Adult epithelium |
| AF | Adult fibroblast |
| AI | Artificially inseminating |
| ANOVA | Analysis of variance |
| ARMS | Amplification refractory mutation system |
| ART | Assisted reproductive techniques |
| AS | Allele-specific |
| ATP | Adenosine triphosphate |
| ATPase6 | Adenosine triphosphate enzyme subunit 6 |
| ATPase8 | Adenosine triphosphate enzyme subunit 8 |
| Bl | Blastocyst |
| BME | Basal medium Eagle |
| Bp | Base pair |
| Br | Brain |
| BSA | Bovine serum albumin |
| Cb | Cerebellum |
| CD | Common deletion |
| cDNA | Complementary DNA |
| CMA | Chaperon-mediated autophagy |

| | |
|---|---|
| Co | Cotyledon |
| CO I | Cytochrome c oxidase subunit I |
| CO III | Cytochrome c oxidase subunit III |
| COCs | Cumulus oocyte complexes |
| CT | Cytoplasmic transfer |
| Cu | Cumulus |
| D-loop | Displacement loop |
| DNA | Deoxyribonucleic acid |
| dNTP | Dinucleotides |
| DPC | Days post coitum |
| EB | Early bud |
| ECNT | Embryonic cell nuclear transfer |
| EGFP | Enhanced green fluorescent protein |
| Em | Embryo |
| Fe | Fetus |
| FF | Fetal fibroblast |
| Gapdh | Glyceraldehyde 3-phosphate dehydrogenase |
| gDNA | Genomic DNA |
| GV | Germinal vesicle |
| GVT | Germinal vesicle transfer |
| hCG | Human chorionic gonadotropins |
| Ht | Heart |
| Id | Identification |

| | |
|---|---|
| IDV | Intensity density value |
| In | Intestine |
| IU | International units |
| IVF | In vitro fertilization |
| Kd | Kidney |
| L | Liter |
| LB | Late bud |
| Lg | Lung |
| LH | Luteinizing hormone |
| Lv | Liver |
| MEM | Minimum essential medium |
| mg | Milligrams |
| M-II | Metaphase-II |
| Min | Minutes |
| ml | Milliliters |
| µl | Microlitres |
| µm | Micrometers |
| mM | Millimolar |
| mRNA | Messenger RNA |
| MT | Mutated type |
| MtDNA | Mitochondrial DNA |
| Mt-nd1 | NADH dehydrogenase subunit I |

| | |
|---|---|
| Mt-nd2 | NADH dehydrogenase subunit II |
| Mt-nd3 | NADH dehydrogenase subunit III |
| Mt-nd4 | NADH dehydrogenase subunit IV |
| Mt-nd5 | NADH dehydrogenase subunit V |
| Mt-nd6 | NADH dehydrogenase subunit VI |
| Mu | Muscle |
| NA | Not analyzed |
| NAD | Nicotinamide adenine dinucleotide |
| NADH | Reduced nicotinamide adenine dinucleotide |
| NADH dehydrogenase | Reduced nicotinamide adenine dinucleotide dehydrogenase |
| nDNA | Nuclear DNA |
| Ndufs4 | NADH dehydrogenase (ubiquinona) Fe-S protein 4 |
| ng | Nanograme |
| Ov | Ovary |
| OXPHOS | Oxidative phosphorylation |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| pg | Picogram |
| PGCs | Primordial germ cells |
| Pmol | Picomol |
| PMSG | Pregnant mare serum gonadotropins |
| PNT | Pronuclear transfer |

| | |
|---|---|
| PVA | Polyvinyl alcohol |
| $R^2$ | Coefficient of determination |
| REMS-qPCR | Restriction endonuclease-mediated selective quantitative real-time PCR |
| RFLP | Restriction fragment length polymorphism |
| RNA | Ribonucleic acid |
| RNAase | RNA enzyme |
| ROS | Reactive oxygen species |
| rRNA | Ribosomal RNA |
| RT | Reverse transcription |
| SC | Standard curve |
| SCNT | Somatic cell nuclear transfer |
| Se | Semen |
| Sec | Seconds |
| Sk | Skin |
| SNP | Single nucleotide polymorphism |
| Sp | Spleen |
| SSCP | Single-strand conformation polymorphism |
| St | Stomach |
| Ta | Tail |
| Te | Testicle |
| Th | Thymus |
| Tm | Temperature melting |

| | |
|---|---|
| To | Tongue |
| tRNA | Transfer RNA |
| UC | Umbilical cord |
| w/v | Weight/volume |
| WBC | White blood cells |
| WT | Wild type |

# Introduction

Endosymbiosis is the most intimate form of symbiosis, in which one symbiotic partner (the endosymbiont) is living intracellulary within the second symbiotic partner (the host). This relationship produces a complementation of the host's limited metabolic capabilities by the biochemical versatility of the endosymbiont. It is assumed that symbiosis provides a nutrient rich, sheltered environment but, it is possible to see the process of endosymbiosis more as an enslavement of the endosymbiont than as a mutually beneficial relationship. The most important examples of endosymbiosis are the endosymbiotic acquisition of plasmids and mitochondria, introducing photosynthesis and respiration to eukaryotes (NOWACK and MELKONIAN 2010).

About 4 billion years ago, algae and anaerobic photosynthetic bacteria began to produce oxygen that eventually diffused throughout the planet. At a certain moment, eukaryotic cells, meaning cells with defined nucleus, specialized organelles that contain genetic material, deoxyribonucleic acid (DNA), appeared. These cells were incapable of using oxygen - they were anaerobic. At some time, some of the existing prokaryotic cells began to use oxygen but they were the minority. The increasing oxygen in the water made the environment unfavorable for the majority of anaerobic cells. There is evidence suggesting that at a certain moment, the prokaryotic cells able to use oxygen invaded the anaerobic cells. These invading cells became the mitochondria and after this association the new cells were able to utilize the oxygen for the purpose of energy production, with at least

two different organelles, the nucleus and the mitochondria. These new cells, produced by an endosymbiotic prokaryotic process, were the common ancestors of the actual eukaryotic cells. This hypothesis, proposed by a scientist, Lynn Margulis, is based on the fact that the mitochondria contain genetic material (DNA) similar to bacterial DNA with a genetic code different from nuclear DNA (ALBERTS *et al.* 2002; KURLAND and ANDERSSON 2000; LANE 2011; MARGULIS 1975).

The eukaryotic cells produce the energy by two pathways. The anaerobic pathway, the glycolysis that occurs in the cytosol and the aerobic pathway, that occurs inside the mitochondrion and includes the reactions produced in the Krebs cycle and the oxidative phosphorylation. The aerobic pathway is far more efficient than the anaerobic pathway producing 16 times more energy. Either or both respiratory pathways can be used depending of the energy requirements of the cell. The mitochondrion has an outer membrane and an inner membrane. The inner membrane forms series of infolding known as cristae. Inserted to the inner membrane, the enzymatic subunits of the electron transport chain produce the oxidative phosphorylation (OXPHOS), the main source of adenosine triphosphate (ATP) of the cell. In normal cells, OXPHOS reactions produce also reactive oxygen species (ROS). These ROS are harmful to mtDNA and can produce mutations. These harmful effects of ROS production are exacerbated in the process of aging (WALLACE 2005; WILDING *et al.* 2009; WILDING *et al.* 2005).

The maternal heredity of mtDNA is the reason of homoplasmy at beginning of mammal's life; homoplasmy means cells harboring the same mitochondrial DNA. This condition can be changed by many factors like mutations or artificial manipulations. This new condition is called heteroplasmy, which means cells harboring more than one sort of mtDNA (EVANS *et al.* 1999; JANSEN and DE BOER 1998).

Nowadays, there is a concern about the effects of the natural heteroplasmy produced in some sickness, the natural heteroplasmy produced by aging and the artificial heteroplasmy produced by intensive utilization of assisted reproductive technologies. By understanding the different patterns of mitochondrial DNA segregation in natural and artificial conditions, we can obtain insight if the heteroplasmy produced at the beginning of the embryonic life can produce deleterious effects in the development and growth of mammals.

# Chapter I

# Literature review

### 1.1. Mitochondria and mitochondrial deoxyribonucleic acid

It is believed that mitochondria originated by an endosymbiotic process between a α-proteobacteria and the ancestors of eukaryotic cells. In this process the cells acquired the oxidative phosphorylation for the production of energy. These organelles have their own mtDNA, ribonucleic acid (RNA), and protein synthesis system (CUMMINS 2001; WALLACE 2005).

Mitochondria are cellular organelles whose primary function is the production of energy (ATP) mainly by OXPHOS. Also, mitochondria host several metabolic pathways, like the Krebs cycle, β-oxidation and lipid and cholesterol synthesis. Moreover, mitochondria participate in cell signaling for apoptotic cell death (CUMMINS 2001; SCHAPIRA 2006), participate in intracellular $Ca^{2+}$ homeostasis, and synthesis of steroids, heme and iron-sulfur clusters (NICHOLLS 2002; WALLACE 2005; ZOROV *et al.* 2007). Finally, mitochondria are the main site of production of ROS (FRIDOVICH 1995).

The eukaryotic cells have two genomes, the nuclear DNA and the mtDNA (EVANS *et al.* 1999). The mitochondrial function is controlled by around 1500 nuclear genes and 37 mitochondrial genes (WALLACE 2005). The vertebrate mitochondrial genomes are closed circular molecules of about 16 – 18 kb containing 37 genes and the D-loop, i.e. the non-coding regulatory region of mtDNA. Mitochondrial genome contains 22 tRNAs, 2 rRNAs and 13 mRNAs that encode proteins that are structural subunits of the OXPHOS enzyme complexes (ANDERSON *et al.* 1981; SMITH *et al.* 2002; WALLACE 2005). The genes (approximately 80) that encode the remaining proteins of the OXPHOS complexes are located in the nucleus and, in contrast to the maternally inherited mitochondria, their inheritance follows the Mendelian laws (TAMASSIA *et al.* 2004).

MtDNA represents approximately 1% of the cellular DNA (GRAZIEWICZ *et al.* 2006; PIKÓ and MATSUMOTO 1976) and consists mostly of tightly packed exons without introns between genes. The mutation rate of mtDNA is 20 times greater than nuclear DNA, possibly due to the lack of histones (JANSEN and DE BOER 1998). While nuclear DNA (nDNA) is confined exclusively to the nucleus, with only two copies per cell, and transmitted from both parents, mtDNA is confined to the mitochondria, with hundred to thousand copies per cell, and transmitted exclusively from the mother (POULTON and MARCHINGTON 2002; WALLACE 1995). This maternal inheritance was supported by experiments made in mice and cattle. Kaneda *et al*. (1995) observed the disappearance of the paternal mtDNA after the pronucleus stage in intraspecific mouse crosses. Sutovsky *et al*. (1996) observed the extinction of MitoTracker-labeled sperm mitochondria at the third

mitotic cycle after in vitro fertilization in cattle (KANEDA *et al.* 1995; SUTOVSKY *et al.* 1996).

In somatic cells, the number of mitochondrial nucleoids per mitochondrion ranges from 1 to more than 10, the copy numbers of mtDNA per mitochondrion ranges from 1 to 15 and the number of mitochondria per cell range between $10^3$ and $10^4$, correlating with each particular cell's requirement of OXPHOS (SATOH and KUROIWA 1991; SPIKINGS *et al.* 2006; YIN *et al.* 2004).

During oocyte maturation, from primordial germ cells to preovulatory oocyte, the number of mitochondria and mtDNA molecules (copies) increase reaching the highest number of copies in the mature preovulatory oocyte. At this stage, mitochondria are thought to be haploid, i.e. one copy by organelle (JANSEN and DE BOER 1998; SMITH and ALCIVAR 1993; SPIKINGS *et al.* 2006). In mature mouse oocytes, mtDNA copy number ranges between 119,000 (PIKÓ and TAYLOR 1987) and 159,000 (STEUERWALD *et al.* 2000). In mature bovine oocytes, 370,000 copies of mtDNA has been reported (TAMASSIA *et al.* 2004). In human oocytes, reports of mtDNA copy number have varied from 193,000 (REYNIER *et al.* 2001) to 314,000 (STEUERWALD *et al.* 2000), and others reported wide ranges from 10,000 to 700,000 (SANTOS *et al.* 2006).

In mammalian cells the energy is available in the form of adenosine triphosphate (ATP). ATP is produced by both anaerobic and aerobic respiration. Anaerobic respiration is

cytoplasmic and involves the breakdown of pyruvate to lactic acid. Aerobic respiration occurs within mitochondria and involves the complete oxidation of pyruvate to carbon dioxide and water (WILDING *et al.* 2005).

The mammalian oocytes posses the enzymes for both energy productions pathways, the aerobic and anaerobic respiration (WILDING *et al.* 2005). There are evidences that mitochondrial metabolism (aerobic respiration) predominates during early oogenesis in the follicle cells surrounding the oocyte because the oocyte itself reside in a relatively hypoxic environment (GOSDEN and BYATT-SMITH 1986). In other experiments, the correlations between the vascularity of growing follicles, the dissolved oxygen content of follicular fluid, and the quality of oocytes from these follicles, suggest that oxidative phosphorylation plays an important role in oogenesis (VAN BLERKOM 2000; VAN BLERKOM *et al.* 1997).

The rate of oxygen consumption increases in the oocyte after the luteinizing hormone (LH) surge, and this could be interpreted like increase in mitochondrial metabolism (MAGNUSSON *et al.* 1977; MAGNUSSON *et al.* 1981). Also, after LH surge there is an increase in lactic acid suggesting anaerobic respiration (HILLENSJÖ 1976; NILSSON 1974; TSAFRIRI *et al.* 1976) but, when the anaerobic respiration is blocked with iodoacetate the oocyte maturation continues (MAGNUSSON *et al.* 1977). However, when the aerobic respiration is blocked the oocyte maturation is arrested (GWATKIN and HAIDRI 1974; HU *et al.* 2001; ZEILMAKER and VERHAMME 1974).

During the early preimplantation development there is no mitochondrial replication, and these organelles have poorly formed cristae, suggesting that aerobic respiration is low during this stage (EBERT *et al.* 1988; MEIRELLES and SMITH 1998; PIKÓ and TAYLOR 1987). At blastocyst stage the mitochondrial cristae become more compact, the utilization of glucose is increased and the mitochondrial replication begins. All these events suggest that aerobic respiration is upregulated at this embryonic phase (BARNETT and BAVISTER 1996; GARDNER *et al.* 1993; LEESE 1995; LEESE and BARTON 1984; LEESE *et al.* 1993; MARTIN and LEESE 1995).

In summary, since the ancestors of mitochondria were introduced into anaerobic cells by an endosymbiotic process, these cells acquired the capacity of energy production by two pathways: an older anaerobic production of energy (cytosolic) and a new aerobic production of energy (mitochondrial). This new production of energy was accompanied also by a production of ROS. Resembling the bacteria, the mitochondria have their own circular mtDNA inside structures called nucleoids. In the mammalian cells, the mature preovulatory oocyte has the highest number of mtDNA molecules (acquired during oogenesis). Possibly, this is a genetic mechanism to ensure the distribution of mtDNA molecules in the cells when the replication of mtDNA is arrested in the early embryo. The oocyte has the enzymes of the two energy production pathways. However, the results of some experiments have indicated that energy production in oocyte maturation relies more on the mitochondrial energy production pathway than on the cytosolic energy production pathway.

## 1.2. Heteroplasmy in mitochondrial deoxyribonucleic acid

It is assumed that all cells from an animal contain the same mtDNA haplotypes, a condition known as homoplasmy. Homoplasmy can be modified by many factors during the life by enabling the production of new mtDNA haplotypes, a new condition known as heteroplasmy (JANSEN and DE BOER 1998). Now, population genetic studies in humans have shown that mitochondria from individuals can harbor heteroplasmy and this is common and widespread phenomenon across a diversity of taxa. Consequently, the possibility exists that a heteroplasmic female could transmit heteroplasmic mtDNA to her offspring. However, a rapid segregation of heteroplasmy has been observed in a few generations suggesting the possibility that a genetic bottleneck exists during the transmission of mtDNA (WOLFF *et al.* 2011).

The mitochondria generate most of the endogenous ROS of the cell (WALLACE 2005). These ROS are generated at very low levels during the normal function of the mitochondrial respiratory chain but it has been proposed that ROS can cause mutations in mtDNA (TAYLOR and TURNBULL 2005). MtDNA diseases are known to be caused by mutations in both mitochondrial or nuclear genes (POULTON and MARCHINGTON 2002; SPIKINGS *et al.* 2006). The mtDNA has a very high mutation rate and, when a new mutation appears and remains, the mixed intracellular population of mtDNA is known as heteroplasmy (WALLACE 2005).

The mutation rate in the mtDNA is 20 times greater than nDNA, probably because the mtDNA is close to the site of ROS production (cristae) and the absence of histones (WALLACE *et al.* 1987). Most inherited mtDNA mutations are insertions. Spontaneous mtDNA mutations are primarily deletions (WALLACE 2005). Approximately, 0.25% of the human population carries pathogenic mtDNA mutations. These may cause epilepsy, liver failure, cardiomyopathy, or sudden death; or milder disorders related with age like deafness, diabetes and loss of vision (POULTON *et al.* 2010). MtDNA mutations seem to play an important role in human evolution, degenerative diseases and aging (WALLACE 1995).

During cellular division, mitochondria are randomly distributed among daughter cells. If the parent cell is heteroplasmic, the proportion of wild-type and mutant mtDNA will vary among the daughter cells. If the cellular division continues, the law of probability dictates that the mtDNA population in the daughter cells drifts toward homoplasmy for normal or for mutant mtDNA (segregation) (HOFHAUS *et al.* 2003).

In conclusion, the condition of homoplasmy from animal cells could be modified easily because the mtDNA lacks protective mechanisms like histones, and mtDNA molecules are near ROS production site. These ROS can produce mtDNA mutations, and given the case these mutations remain inside the cells, they can produce heteroplasmy. If a condition of heteroplasmy exists in cells and the mitotic divisions continue, this condition could drift to homoplasmy of normal or mutated mtDNA.

### 1.3. Heteroplasmy in assisted reproductive technologies

In natural fertilization mtDNA is transmitted in a homoplasmic fashion (MONNAT *et al.* 1985; SPIKINGS *et al.* 2006). However, nowadays the maternal transmission of mtDNA is altered by the increased utilization of assisted reproductive techniques (ART). This has led to the incorporation of exogenous mtDNA (heteroplasmy) in both reconstructed oocyte and embryo with transmission to the offspring at varying degrees (ST JOHN 2002). Techniques like cytoplasmic transfer (CT), germinal vesicle transfer (GVT), pronuclear transfer (PNT) and somatic cell nuclear transfer (SCNT) can produce heteroplasmy in living organisms at different levels (SPIKINGS *et al.* 2006).

In the nuclear transfer technique a whole cell containing both nucleus and cytoplasm is introduced into an enucleated recipient oocyte by electrofusion. The most common donor cells utilized are embryonic cells (embryonic cell nuclear transfer or ECNT) like blastomeres or different kinds of somatic cells (somatic cell nuclear transfer or SCNT). During this process nuclear and mitochondrial DNA from the donor cell are transferred to the recipient oocyte enabling the cloned progeny to harbor mtDNA from donor and recipient cytoplasms and, thereby, producing heteroplasmy (EVANS *et al.* 1999; SPIKINGS *et al.* 2006).

There are three possible outcomes in the initial heteroplasmy produced after the electrofusion of donor and recipient cells in SCNT experiments: homoplasmy of recipient

oocyte mtDNA, homoplasmy of donor somatic cell mtDNA or heteroplasmy with mix of both mtDNAs in different proportions (EVANS *et al.* 1999).

The assessment of the mtDNA content after nuclear transfer has produced different levels of heteroplasmy. Some experiments obtained homoplasmic results with mtDNA coming only from the recipient oocyte. Evans and colleagues analyzed tissues from "Dolly", the first animal cloned from by SCNT, and other nine sheep produced by fetal SCNT. They found mtDNA homoplasmy from the recipient oocytes in all ten animals (EVANS *et al.* 1999). Takeda and colleagues analyzed different somatic tissues in calves produced by ECNT (blastomeres from morula) and also obtained homoplasmy for recipient oocyte (TAKEDA *et al.* 1999). In bovine intraspecific SCNT experiments utilizing cumulus donor cells showed that the donor mtDNA disappeared during early development at 16 cell stage (DO *et al.* 2001). Hiendleder and colleagues in bovine intraspecie SCNT experiments observed homoplasmy in somatic and placental tissues from fetuses of 80 days (HIENDLEDER *et al.* 2004).

Other nuclear transfer experiments showed only heteroplasmic results. In bovine intraspecific ECNT experiments utilizing blastomere donor cells obtained heteroplasmy in 3 clones ranging from 0.4 to 18% (STEINBORN *et al.* 1998). Hiendleder and colleagues, also using blastomeres donor cells, obtained heteroplasmy in blood and cloned progenies, ranging between 2.98 and 57.43 % from donor cell mtDNA (HIENDLEDER *et al.* 1999). In bovine SCNT experiments with ear fibroblasts donor cells showed heteroplasmy cloned

progeny (HAN *et al.* 2004). St. John and Schatten in interspecie primate ECNT experiments analyzed white blood cells from offspring and found triparental heteroplasmy using blastomeres from IVF of Indian *Macaca Mulatta* oocytes and sperm from a Chinese *Macaca Mulatta*, and fused with Indian *Macaca Mulatta* enucleated oocytes (ST. JOHN and SCHATTEN 2004). In porcine SCNT experiments, fetal fibroblasts were used as donor cells obtaining heteroplasmy in blood and ear samples from clone progeny, ranging between 0.1 – 1% (TAKEDA *et al.* 2006). Takeda and colleagues in intraspecific bovine SCNT experiments analyzed somatic tissues from cows, calves and fetuses and found heteroplasmy around 7.6% in 1/6 clones and < 5% in 5/6 clones. Also they sampled alive clones (blood and hair roots) and found heteroplasmy ranging between 17 and 51% in 4/12 clones and < 5% in 8/12 clones (TAKEDA *et al.* 2008). In interspecific goat-ovine SCNT experiments using *Capra hircus* fetal fibroblasts donor cells and *Ovis aries* enucleated oocytes observed heteroplasmy in cloned embryos from 1 cell (1%) to morula (2.2%) and with significant reduction in blastocyst stage (0.012%) (MA *et al.* 2008).

Other researchers found homoplasmic and heteroplasmic results in the same nuclear transfer experiments. Steinborn and colleagues utilized different bovine donor cells like fetal fibroblasts (FF), adult epithelium cells (AE) and adult fibroblasts (AF). They analyzed somatic tissues from fetus, and they obtained heteroplasmy levels of 1-4% (FF), 0.7-0.9% (AF) and 0% in some tissues (STEINBORN *et al.* 2000). Meirelles and colleagues, in bovine interspecies ECNT experiments observed a decrease in heteroplasmy from embryos from 1 cell until blastocyst stage. They found different levels of heteroplasmy in extrafetal and

fetal tissues. Also, they observed an homoplasmic calf from recipient oocyte mtDNA (MEIRELLES *et al.* 2001). Steinborn and colleagues (in intra- and interspecific SCNT experiments) utilized *Bos indicus* mural granulosa like donor cells and they obtained heteroplasmy levels between 0.6-2.8 % in 4/11 indicus/taurus cattle and 1% in 7/9 indicus/indicus cattle (STEINBORN *et al.* 2002). In bovine intraspecific SCNT experiments homoplasmic and heteroplasmic embryos were obtained utilizing cumulus like donor cells (DO *et al.* 2002). Takeda and colleagues in bovine SCNT experiments found homoplasmy in embryos and 2 fetuses, and 1 calf was heteroplasmic ranging between 6-40% (TAKEDA *et al.* 2003). Hiendleder and colleagues in bovine SCNT experiments analyzing somatic and placental tissues from 80 days fetuses found homoplasmy in 10 fetuses and heteroplasmy in 1 fetus (0.5 – 0.7%) (HIENDLEDER *et al.* 2003). Lloyd and colleagues performed intraspecific ovine SCNT experiments by using ovine fetal fibroblasts which were unmodified (complete) (+), partially depleted (pd) and residually depleted (r) of mtDNA. They analyzed embryos (2-cell to blastocyst stages) and found from 0.03 to 8.72% of heteroplasmy in complete mtDNA embryos (+) and from 0.00 to 0.02% in residually mtDNA-depleted embryos (r) (LLOYD *et al.* 2006). In intraspecific ovine SCNT experiments were used fetal fibroblasts like donor cells and somatic and placental fetal tissues showed 7/12 fetuses to be heteroplasmic, of which 6 fetuses ranging between 0.1 and 0.9% and 1 fetus ranging between 6.8 and 46.5% (BURGSTALLER *et al.* 2007). Ferreira and colleagues in interspecific bovine SCNT and ECNT experiments fused *Bos indicus* adult fibroblast and blastomere donor cells to *Bos taurus* slaughterhouse oocytes. They found heteroplasmy in embryos (blastomeres) produced by different donor cells. Also, they

found homoplasmy in the somatic tissues from a newborn calf (FERREIRA *et al.* 2007). Sansinema and colleagues analyzed the consequence of caprine ooplasm transfer into bovine enucleated oocyte, followed by SCNT. They analyzed embryos at hatched blastocyst stage and they found heteroplasmy in 83% of embryos and homoplasmy in 17% of embryos (SANSINEMA *et al.* 2011).

Only a few nuclear transfer experiments have been reported, showing homoplasmy for the mtDNA donor cell: (i) a bovine intraspecific nuclear transfer experiments (SMITH *et al.* 2000), and (ii) a rabbit-panda interspecific nuclear transfer experiments (CHEN *et al.* 2002).

CT involves the injection of supplementary cytoplasm from another oocyte and sperm into the recipient oocyte (SPIKINGS *et al.* 2006). CT frequently uses a young oocyte as donor, which supposedly contains no defective mtDNA and other important cytoplasmic components (COHEN *et al.* 1997). It has been shown in human offspring that CT can produce heteroplasmy (BRENNER *et al.* 2000).

GVT and PNT are also other techniques proposed to resolve problems with older oocytes. In these techniques a nucleus from a germinal vesicle (GV) oocyte or the two pronuclei from a zygote are transferred to an enucleated oocyte (LIU *et al.* 1999). These two techniques could produce heteroplasmy (SPIKINGS *et al.* 2006).

In short, the increased utilization of ART has produced heteroplasmy in mammals to different degrees. The main outcomes observed after SCNT techniques were homoplasmy from mtDNA recipient oocytes, and heteroplasmy of mtDNAs from donor and recipient cells to different degrees. A rare outcome from SCNT experiments was the homoplasmy from mtDNA donor cells observed only in a few experiments. ART techniques like CT, GVT and PNT can also produce heteroplasmy.

### 1.4. Heteroplasmy produced by aging

Aging is characterized by time dependent decline in physiological functions (MANDAVILLI *et al.* 2002). It is difficult to explain all the mechanisms of aging by a single theory, but there is a general consensus that, a time dependent accumulation of intracellular oxidative damage plays an important role in the process of aging. This is the basis of the free-radical theory of aging (HARMAN 1956; TARÍN 1996). This theory proposed that most aging changes are due to molecular damages caused by free radicals, which are highly reactive because they are atoms or molecules that contain an unpaired electron. Aerobic metabolism generates the superoxide radical ($O_2^{\cdot -}$), which is metabolized by superoxide dismutase to form hydrogen peroxide ($H_2O_2$) and oxygen. Hydrogen peroxide can go on to form the extremely reactive hydroxyl radical ($^{\cdot}OH$) (TROEN 2003). The free-radical theory of aging is essentially a mitochondrial theory because the OXPHOS produced in the mitochondria is the main source of ROS (PARTRIDGE and GEMS 2002). This theory involves the damaging role of ROS on mitochondrial molecular components such as lipids,

proteins, and DNA. In particular, the mtDNA is more susceptible to mutations like point mutations (single nucleotide substitutions) or large size rearrangements (deletions, insertions) (CASSANO *et al.* 2004).

The electron transport chain consumes more than 90% of the oxygen introduced into the cell, from which about 1-5% is converted into superoxide ($O_2^{\cdot -}$) even during normal physiological state. This basal rate of $O_2^{\cdot -}$ production may be increased in pathological conditions resulting in elevated oxidative stress (MANDAVILLI *et al.* 2002). ROS are produced continuously in mitochondria due to leakage of high-energy electrons along the electron transport chain. MtDNA lacks both protective histones and DNA repair activity, and can be a primary target of oxidative damage (HAMATANI *et al.* 2004).

Decreased activity in mitochondria has been found in animal tissues with aging such as diaphragm, skeletal muscle, heart and liver. Two observations point to mitochondrial DNA as the cause of decreased activity. First, the effects were more pronounced for proteins encoded by mitochondrial DNA (Complex I and IV), while Complex III was less affected (with only one mitochondrial encoded subunit) and Complex II was unaffected (solely encoded by nuclear DNA) (HOFHAUS *et al.* 2003).

The reduction of OXPHOS produced by aging could result of loss of mitochondria, reduced mtDNA copy number, reduced mtDNA transcription, impaired coupling between substrate oxidation and ATP synthesis and mtDNA mutations (MCINERNY *et al.* 2009).

As mtDNA encodes thirteen proteins of the respiratory chain, mutations in any of these mitochondrial genes compromise the mitochondrial OXPHOS. According with the mitochondrial theory of aging, mitochondrial mutations accumulate with time resulting in OXPHOS decline in a number of tissues (CORRAL-DEBRINSKI *et al.* 1992a; GADALETA *et al.* 1992; GERHARD *et al.* 2002; KHAIDAKOV *et al.* 2005; MICHIKAWA *et al.* 1999; NEKHAEVA *et al.* 2002; ZHANG *et al.* 1997).

There are mtDNA deletions ranging from 6 to 10 422 bp in size. The most frequent and best-characterized mtDNA deletion is 4977 bp in length (the common deletion) in humans (HOFHAUS *et al.* 2003), 4974 bp in mouse and 4834 bp in rat (TANHAUSER and LAIPIS 1995). This common deletion is considered to be a marker for mutations in the mitochondrial genome (HOFHAUS *et al.* 2003). A 13-base-pair direct repeat (humans) has been observed upstream from both breakpoints of this common deletion. Relative to the direction of heavy-strand replication, the first repeat was retained and the second repeat was deleted suggesting a slip-replication mechanism (SHOFFNER *et al.* 1989). In other species, the direct repeats are 15 base-pair in mouse and 16 base-pair in rats (TANHAUSER and LAIPIS 1995). It has been proposed that a single-oxygen-generation is responsible for base damage and subsequent strand break formation (BERNEBURG *et al.* 1999).

The mtDNA common deletion has been studied by many researchers. In human somatic tissues sampled from birth to 87 years, this common deletion was detected in all tissues from adults but not in tissues from infants (LINNANE *et al.* 1990). Cortopassi and

Arnheim (1990) found the same deletion in heart, muscle and brain from human normal adults, which was not observed in fetal heart or brain (CORTOPASSI and ARNHEIM 1990). Corral-Debrinski *et al*. (1992) observed a significant increase in accumulation of mtDNA common deletion (4977 bp) in brain samples from elderly people (CORRAL-DEBRINSKI *et al.* 1992a). Simonetti *et al*. (1992) estimated a 10,000-fold increase in mtDNA common deletion during the normal human lifespan. The maximum percentage was found in muscle (0.1%). Undividing tissues (skeletal muscle and heart) contained higher proportions of mtDNA common deletion than dividing tissues (liver) (SIMONETTI *et al.* 1992). In another experiment, human tissues from liver and muscle were analyzed. The 4,977 bp deletion started to appear in the second and third decades of life. At the age of 70 years old, muscle showed 0.06% of this deletion and liver 0.0076% (LEE *et al.* 1994). The estimated percentage of mtDNA common deletion in elderly people range from 0.007 to 0.1% in heart (CORRAL-DEBRINSKI *et al.* 1992b; CORTOPASSI and ARNHEIM 1990), 0.001 to 0.12% in brain (CORRAL-DEBRINSKI *et al.* 1992a; SOONG *et al.* 1992) and 0.02 to 0.1% in muscle (COOPER *et al.* 1992; SIMONETTI *et al.* 1992). Tanhauser and Laipis (1995) analyzed eight mtDNA deletions in mouse somatic tissues, included the 4974 bp deletion similar than mtDNA common deletion in humans (4977 bp). They found that deletions were most abundant in liver, moderately abundant in kidney and lung, and either low or absent in brain, heart, muscle, tail, skin and blood (TANHAUSER and LAIPIS 1995).

No correlation was found between the presence of mitochondrial common deletion and age in human oocytes and embryos (BARRITT *et al.* 1999; BARRITT *et al.* 2000;

BRENNER *et al.* 1998; CHEN *et al.* 1995; MÜLLER-HÖCKER *et al.* 1996). Only Chan and colleagues found more incidence of mtDNA common deletion in older unfertilized human metaphase II oocytes (≥ 35 years of age) and lower mtDNA copy number compared with young unfertilized M-II oocytes (CHAN *et al.* 2005).

In some experiments more mtDNA common deletions have been found in oocytes compared to embryos (humans) (BRENNER *et al.* 1998). Further, there are more deletions in granulosa cells from women older than 38 years (SEIFER *et al.* 2002). A significant increase was found also in the frequency of deleted mtDNA in unfertilized human oocytes (HSIEH *et al.* 2002). Hsieh et al. (2004) have reported lower expression levels of ATPase6, CO III, and Mt-nd3 genes in unfertilized human oocytes and in arrested embryos harboring the common deletion (HSIEH *et al.* 2004). Gibson *et al*. (2005) reported a significant increase in the frequency of mtDNA common deletion in oocytes with gonadotropins stimulation and embryos from rhesus macaques (GIBSON *et al.* 2005). In all these experiments, the common deletion was quantified by semi-quantitative nested PCR, indicating that the results are qualitative in nature, i.e. deleted (+) or not deleted (-). Results were reported as number of oocytes or embryos affected with the mtDNA common deletion within a sample population and expressed as percentage. None of the experiments described above utilized the percentage of deleted copies within the total copies of mtDNA of each oocyte, embryo or somatic cell.

In summary, the accumulation of oxidative damage by ROS throughout life forms the basis of the free-radical theory of aging. ROS are produced mainly by OXPHOS in the cristae of mitochondria. These ROS can produce oxidative damage in the mtDNA and generate mutations as insertions, deletions and point mutations. The mtDNA common deletion is the most studied mtDNA deletion, and has been correlated with aging in somatic tissues but not in germinal tissues. Moreover, a greater presence of common deletion has been found in oocytes than embryos. The results of these experiments are mainly qualitative, with no real quantification of percentage of common deletion within the cells.

### 1.5. The theory of the bottleneck

The concept of genetic bottleneck was developed by segregation analysis of mitochondrial polymorphisms in Holstein cows (ASHLEY *et al.* 1989; HAUSWIRTH and LAIPIS 1982; LAIPIS *et al.* 1988). Hauswirth and Laipis (1982) observed two mitochondrial genotypes within one Holstein cow maternal lineage (probably due to a neutral or silent mutation). They observed a shift from one mitochondrial genotype to another in two or maximum five generations.(HAUSWIRTH and LAIPIS 1982). Laipis and colleagues (1988) found different levels of two polymorphic mtDNA in tissues of three bovine siblings; two siblings had approximately the same proportion of heteroplasmy whereas significant difference was found in the heteroplasmy of the third sibling (3-fold). The heteroplasmy was due to a single guanine position in the mtDNA. This heteroplasmy was explained by unequal distribution of the mtDNA in early mitotic embryonic divisions (LAIPIS *et al.*

1988). Ashley and colleagues (1989) analyzed the transmission of a heteroplasmic mtDNA mutation in Holstein cows, and they observed a rapid rate of mtDNA segregation that returned to homoplasmy in only two or three generations. They suggested a genetic bottleneck in mtDNA inheritance during oocyte maturation or early embryogenesis to explain this phenomenon (ASHLEY *et al.* 1989).

To explain this rapid segregation of mtDNA a genetic bottleneck for mtDNA in the female germline or early embryo has been proposed. There are two hypothesis that can explain this phenomenon: 1) Selection and replication of small subpopulations of mtDNA templates during the mitochondrial amplification (100-fold) since primary oocyte until mature oocyte can produce rapid shifts in mtDNA genotype in only one generation (HAUSWIRTH and LAIPIS 1982). 2) Uneven distribution of the mtDNA in the mitotic divisions of inner cell mass at blastocyst stage could explain the rapid segregation of the mtDNA (LAIPIS *et al.* 1988).

More recently with different mouse models of experiments many researchers have been proposed different theories of the bottleneck at different stages of development in the pre-natal and post-natal life (CAO *et al.* 2007; CAO *et al.* 2009; CREE *et al.* 2008; FAN *et al.* 2008; JENUTH *et al.* 1996; MEIRELLES and SMITH 1997; WAI *et al.* 2008).

Jenuth and colleagues created heteroplasmic mice and showed that mitochondrial segregation occurred by random genetic drift in early oogenesis in the small population of

mtDNAs in oogonia and they were approximately 200. To analyze the mtDNA segregation they used single-cell PCR techniques to measure the different proportions of mtDNA at different stages of oogenesis. They compared the heteroplasmy in mature oocytes and F1 offspring from the same founder females. They did not observe significant differences in heteroplasmy and in the coefficients of variation. They also compared the heteroplasmy between primary oocytes from immature heteroplasmic females (postnatal day 2-3) and mature oocytes from adult mice. The coefficient of variation was similar. They also compared primary and mature oocytes from the same animal and the coefficient of variation was almost identical. They also analyzed the mtDNA genotypes in primordial germ cells and they found that the coefficient of variation in these cells was very small. Summarizing all these results, they concluded that segregation of mtDNA must occurred during expansion of the oogonial population, in early oogenesis and is essentially completed when the primary oocyte population has differentiated (JENUTH *et al.* 1996).

Meirelles and Smith (1997) produced heteroplasmic mouse progeny derived by transplantation of pronuclear stage karyoplasts to enucleated zygotes with different mtDNA. After reconstruction of zygotes were obtained 19% of mtDNA from karyoplast origin. Most progeny of reconstructed zygotes contained less mtDNA of karyoplast origin and produced only homoplasmic first generation progeny. However, one founder heteroplasmic female had elevated levels of tissue heteroplasmy and produced first heteroplasmic progeny. These heteroplasmic females produced new generations with

constant levels of heteroplasmy explained by persistent intraorganellar heteroplasmy (MEIRELLES and SMITH 1997).

Cao and colleagues (2007) estimated by quantitative real time PCR the mtDNA copy number in single zygotes and single cells of wild-type mouse embryos from two cells to 6.5 days post coitum (dpc), to know if there was a reduction of mtDNA content in the precursor of germ cells. The results indicated that amount of mtDNA was constant in the cells of embryos at early cleavage stages. The average of mtDNA in single cells was 1.27 to 1.86 x $10^3$. They concluded that there is not a bottleneck in antecedents of germ cells. Later, they analyzed mtDNA copy number in primordial germ cells (PGCs) (identified by endogenous alkaline phophatase activity) from 7.5 to 13.5 dpc. The average of mtDNA copies did not vary among the different developmental stages. 95% of the PGCs analyzed harbored more than 953 mtDNA molecules. Also, they found more than 100 mitochondria in a single PGC. After, they measured the mtDNA copies since primary oocyte stage (5.16 x $10^3$ copies) until mature oocyte stage (1.57 x $10^5$ copies). They observed substantial mtDNA expansion during early oocyte maturation. Finally, they analyzed the number of mtDNA copies in single somatic cells of 7.5 to 13.5 dpc embryos. They observed marked differences in somatic cells of different stages. Large somatic cells had a much lower mtDNA amount than large PGCs. 54.9% of somatic cells possessed less than 600 mtDNA copies. Summarizing all these results, they concluded that the mitochondrial bottleneck is not due to a drastic decline in mtDNA copy number in early oogenesis but rather to a small effective number of segregation units for mtDNA in mouse germ cells (CAO *et al.* 2007)

and the transmission of mtDNA depends on mitochondrion as its vehicle (ST JOHN *et al.* 2010).

Cree and colleagues (2008) utilized Stella-GFP transgenic mice to isolate PGCs. They found a mean of 450 mtDNA copies per PGC at 7.5 dpc and a mean of 1100 – 2200 mtDNA copies between 8.5 and 14.5 dpc. The reduction of mtDNA content at 7.5 dpc was suggested to be the cause of the bottleneck (CREE *et al.* 2008).

Wai and colleagues (2008) isolated embryonic germ cells from heteroplasmic mice expressing enhanced green fluorescent protein (EGFP) and they made single-cell quantification of mtDNA copy number and heteroplasmy. They detected significant changes in mtDNA copy number during early oogenesis between E8.5 (mean ~ 280, median 145) and E10.5 (mean ~ 2,800, median 2200). By E14.5, when the PGCs have colonized the gonad there are about 6,000 copies of mtDNA per cell. It seems that resumption of mtDNA replication in early development occurs before PGC differentiation. They concluded that there is a severe decrease (700-fold) in mtDNA copy number between the fertilized oocyte until the first PGCs and a moderate increase (10- to 20 fold) during the expansion of PGCs before they colonize the gonad. They did not find significant differences in genotypic variance before decrease and after the increase in mtDNA copy number. They concluded that mitochondrial bottleneck does not occur during embryonic oogenesis. Later, they extend the analysis to the postnatal period and they found different genotypic variance between the groups of germ cells containing more than 10,000 copies of

mtDNA per cell (matured ovulated oocytes, primary oocytes in secondary follicles) and those groups of germ cells containing less than 10,000 copies of mtDNA per cell (PGCs, oogonia, primary oocytes in primordial follicles). They concluded that the genetic bottleneck is due to selective replication of a random population of mtDNA templates during the growth and maturation of the ovarian follicles. According with these results they proposed a model for the transmission of mtDNA: the high mtDNA copies in mature oocytes are a genetic mechanism to ensure distribution of mitochondria to the cells when the replication of mtDNA is arrested in the early embryo. Resumption of mtDNA replication by E6.5 results in 10- to 20-fold increase in mtDNA copy number since the apparition of PGCs until colonization of the gonad at E13.5. Although there is no measurable mitochondrial segregation at this time, a physical bottleneck (~ 200 mtDNA) in the earliest PGCs enables selection against deleterious mtDNA mutations. The genetic bottleneck for less deleterious mtDNA mutations occurs during folliculogenesis in early postnatal life, as result of preferential replication of a subpopulation of mtDNA (WAI *et al.* 2008).

Fan and colleagues (2008) produced cell lines harboring two mitochondrial DNA mutations in two genes involved in the oxidative phosphorylation. The first one was a severe mutation in the NADH dehydrogenase subunit 6 gene (Mt-nd6), which inactivates the oxidative phosphorylation complex I. The other was a mild mutation in the cytochrome c oxidase subunit I gene (COI), which reduces the activity of oxidative phosphorylation complex IV by 50%. Transmitochondrial cybrid cells harboring these mutations were

introduced in the female mouse germ line. They showed that severe Mt-nd6 mutation was selectively eliminated during oogenesis in four generations and the mild COI mutation persisted throughout multiple generations. They proposed that during oogenesis the proto-oocytes with highest percentage of severe mtDNA mutations produce more ROS and could be preferentially eliminated by apoptosis (FAN *et al.* 2008).

Cao and colleagues (2009) manually isolated single PGCs from mice expressing green fluorescent protein (GFP) and measured the mtDNA copy number at different embryonic development stages. They chose two developmental periods, 7.5 and 13.5 dpc to confirm results obtained by the same laboratory (CAO *et al.* 2007). The 7.5 dpc embryos were divided into two groups having morphology of early bud (EB) stage and late bud (LB) stage. The mean of mtDNA copies in single 7.5 dpc EB and LB, 13.5 dpc female and 13.5 male were 1396, 1479, 1747 and 2039, respectively. The coefficient of variation was similar for each group (0.25 – 0.45). There were not significant differences between males and females (13.5 dpc). The somatic cells from gonads (13.5 dpc) had less than half of mtDNA copy number (702) than observed in PGCs. They concluded that: 1) There is no reduction of mtDNA copies in early PGCs; 2) the amount of mtDNA molecules in PGCs (1000 copies) is similar than adult somatic cells and 3) Embryonic somatic cells had lower mtDNA copies than PGCs (CAO *et al.* 2009).

Overall, previous studies support the notion that the mitochondrial bottleneck occurs without reduction of mtDNA copies in early PGCs (CAO *et al.* 2007; CAO *et al.* 2009; WAI

*et al.* 2008). It has been proposed that this bottleneck is due to a small effective number of segregation units for mtDNA in germ cells (~ 200) (CAO *et al.* 2007; CAO *et al.* 2009; JENUTH *et al.* 1996; WAI *et al.* 2008). Some proposed that the bottleneck occurred in early oogenesis (JENUTH *et al.* 1996). Others, that there could be two kinds of bottlenecks: a physical bottleneck in PGCs and a genetic bottleneck in early postnatal oocytes explained by a preferential replication of a subpopulation of mtDNAs (WAI *et al.* 2008). A bottleneck in oogenesis based in the elimination by apoptosis of germ cells harboring severe mtDNA has also been suggested (FAN *et al.* 2008). All these proposals are not mutually contradictory.

## 1.6. Rationale, hypothesis and objectives

### 1.6.1. Rationale

Homoplasmy in the early stages of life could be changed by many factors producing cells harboring different sorts of mitochondrial molecules, which leads to heteroplasmy (EVANS *et al.* 1999). Heteroplasmy can be produced by natural and artificial conditions. Natural heteroplasmy can be produced by the process of aging while artificial heteroplasmy can be produced by the utilization of ART (COOPER *et al.* 1992; CORRAL-DEBRINSKI *et al.* 1992a; CORRAL-DEBRINSKI *et al.* 1992b; CORTOPASSI and ARNHEIM 1990; CORTOPASSI *et al.* 1992; LEE *et al.* 1994; LINNANE *et al.* 1990; SEIFER *et al.* 2002; SIMONETTI *et al.* 1992; SOONG *et al.* 1992; SPIKINGS *et al.* 2006).

Available evidence indicates that mtDNA is constantly undergoing mutation, with clonal expansion or loss of either point mutations or deletions; consequently the concept of homoplasmy is more apparent than real (TAYLOR and TURNBULL 2005).

Fusion enables the stochastic redistribution of soluble and membrane components of normal and defective mitochondria. Fission is useful for the segregation of irreversibly damaged mitochondria and their subsequent elimination by autophagy (TERMAN *et al.* 2010). The ability to select against deleterious mutations in mtDNA has been reported. Somatic cells package multiple mtDNA genomes in a single nucleoid complex. These cells

have a more complex organization of the nucleoids than do oocytes. This fundamental difference suggests that oocytes may be better able to detect and remove defective mtDNA genomes than somatic cells, possibly due in part to the simpler organization of the mtDNA in smaller nucleoids (BOGENHAGEN 2010).

It is now accepted that aging is related to macromolecular damage produced by mitochondrial ROS, mostly affecting long-lived postmitotic cells. The slow accumulation of lipofuscin within lysosomes seems to depress autophagy, resulting in reduced turnover of damaged mitochondria. This produces accumulation of enlarged mitochondria which do not fuse and interchange material with normal mitochondria. The progress of these changes seems to result in enhanced oxidative stress, decreased ATP production, and collapse of the cellular catabolic machinery. All these effects could eventually affect cellular survival (TERMAN *et al.* 2010).

The natural transmission of mutated mtDNA in some sicknesses depends mainly on the mtDNA mutation (insertion, point mutation or deletion) (POULTON and MARCHINGTON 2002). Most inherited mtDNA mutations are insertions. Spontaneous mtDNA mutations are primarily deletions (WALLACE 2005).

When polymorphisms in the mtDNA, like heteroplasmy tracers in SCNT experiments, are utilized, there are more variables that could affect the transmission of

mutated mtDNA: the type of mutated mtDNA introduced with the donor cells, the source of recipient oocytes (different sources of recipient oocytes can increase the chance to produce divergent donor mtDNA segregation patterns), the variety of donor cell (embryonic, fetal or adult cells) and the technique utilized in the introduction of donor cell (microinjection, electrofusion) (BURGSTALLER *et al.* 2007; EVANS *et al.* 1999; SPIKINGS *et al.* 2006).

The segregation of mtDNA molecules tends to follow a pattern of random genetic drift, and it is not possible to predict in which cells or tissues the mutant mtDNA molecules will migrate during early development (ST. JOHN *et al.* 2010). The utilization of NT technique can result in different levels of mtDNA from donor cell and recipient oocyte. The degree of donor mtDNA transmission (in NT experiments) appears to be random and there is no evidence to explain this phenomenon (LLOYD *et al.* 2006).

There is worldwide concern whether heteroplasmy produced by ARTs, i.e. somatic cell nuclear transfer, affects the development of cloned progeny. Also, there is increasing interest in the patterns of segregation of the mtDNA from the donor cell into the reconstructed oocyte and the subsequent distributions during embryo, fetal and post-natal life. Another field of great interest is the heteroplasmy produced by aging in somatic and germinal tissues. There is also concern regarding the increase of mtDNA mutations with the progress of aging in somatic tissues and the deleterious effects of these mutations. Another concern is about the quantity and quality of mutations in the mtDNA from oocytes and

embryos in older females, and if these mutations could be transmitted to the next generations by normal or artificial reproduction in mammals.

### 1.6.2. Hypothesis

The transmission of foreign mutated mtDNA introduced by SCNT follows different segregation patterns during development and growth. The transmission of native mutated mtDNA produced by aging follows different segregation patterns in germinal, embryonic and somatic cells.

### 1.6.3. Objectives

1.6.3.1. To analyze the changes in mtDNA heteroplasmy produced by somatic cell nuclear transfer during development from embryos, to fetuses and adult tissues in cattle.

1.6.3.2. To analyze the changes in mtDNA heteroplasmy caused by aging in (i) adult germinal and somatic tissues, (ii) during oogenesis and early embryogenesis, and (iii) in *in vitro* culture procedures in mice.

# Chapter II

## Neutral segregation of donor cell mitochondria during the development of somatic cell clones in cattle

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## 2.1. Abstract

Since its inception, animal cloning by somatic cell nuclear transfer (SCNT) has been extremely inefficient: only 1-2% of reconstructed oocytes develop to birth. It has been proposed that embryo mortality is due to an incompatibility between nuclear and mitochondrial genes and controversy exists concerning the amount of donor cells mitochondrial DNA (mtDNA) that is inherited by somatic clones (heteroplasmy). Since it remains unclear whether mtDNA segregation is neutral or selective, the purpose of this study was to analyze the transmission of mtDNA from donor somatic cells to embryonic, fetal and adult clones. Fetal fibroblasts carrying a mtDNA mutation (insertion of 66 bp) were fused to host oocytes carrying wild type mtDNA. The presence of mtDNA from the donor cell was analyzed at different stages of development: 17-day-old embryos (n=17); 40-day-old fetuses (n=3); 60-day-old fetuses (n=3); one 240 day-old fetus; and 3 post-natal clones (18-24 months). Every individual clone proved to be heteroplasmic and 99% (103/104) of the analyzed tissue samples were heteroplasmic as well. Most of the analyzed samples (99/104) contained heteroplasmy levels (1.5%) similar to the levels expected during oocyte reconstruction, regardless of development stage, tissue origin or mitotic activity. In contrast, tissues from one single day-40 fetus presented high levels of heteroplasmy (20.9%), indicating rare events of major donor mtDNA contribution. Since most SCNT clones showed negligible changes in heteroplasmy throughout development

and after birth, we conclude that the heteroplasmy produced by nuclear transfer techniques using somatic cells is due to the neutral segregation of the mtDNA.

**Key words:** Bovine SCNT, mtDNA, heteroplasmy, neutral segregation of mtDNA

## 2.2. Introduction

After fertilization, mtDNA is maternally inherited from the oocyte due to the elimination of the sperm mtDNA by a process of ubiquination (BIRKY 1995; SUTOVSKY *et al.* 1999). For this reason, all the cells within an animal should contain the same sort of mtDNA, a condition known as mtDNA homoplasmy. However, homoplasmy can be perturbed during life by the generation of mtDNA mutations and, thereby, the mixing of new haplotypes to the original mtDNA pool, i.e. leading to heteroplasmy (JANSEN and DE BOER 1998). Although the accumulation of significant quantities of pathological haplotypes can cause disease, low levels of heteroplasmy are a common phenomenon during life and often involve silent mtDNA mutations (SMITH *et al.* 2005).

Assisted reproductive techniques (ART) often result in the incorporation of exogenous mtDNA in both reconstructed oocytes and embryos, which can be transmitted to the offspring in different degrees (ST JOHN 2002). ART such as, cytoplasmic transfer (CT) (BRENNER *et al.* 2000; FERREIRA *et al.* 2010), germinal vesicle transfer (GVT) (LIU *et al.* 2003; LIU *et al.* 2001; LIU *et al.* 1999; ZHANG *et al.* 1999), pronuclear transfer (PNT) (SPIKINGS *et al.* 2006) and SCNT (BURGSTALLER *et al.* 2007; DO *et al.* 2001; DO *et al.* 2002; EVANS *et al.* 1999; FERREIRA *et al.* 2007; HAN *et al.* 2004; HIENDLEDER *et al.* 2004; HIENDLEDER *et al.* 2003; LLOYD *et al.* 2006; MA *et al.* 2008; STEINBORN *et al.* 2002; STEINBORN *et al.* 2000; TAKEDA *et al.* 2003; TAKEDA *et al.* 2008; TAKEDA *et al.* 2006) can produce heteroplasmy in different levels (SPIKINGS *et al.* 2006).

Mature oocytes have the highest number of mtDNA copies in the cells. Different mammalian species have been shown to contain between 1.74 to 9.5 X $10^5$ mtDNA copies in mature oocyte (BURGSTALLER *et al.* 2007). A mean of 370,000 mtDNA copies has been found to be present in bovine oocyte but there is large intra and interanimal variation (TAMASSIA *et al.* 2004). In contrast, somatic cells have between 1,000 and 10,000 copies of mtDNA (SPIKINGS *et al.* 2006). In SCNT, a whole somatic cell is fused to an enucleated oocyte leading to a ratio donor/recipient mtDNA of between 0.4 – 0.9% (BURGSTALLER *et al.* 2007; STEINBORN *et al.* 2000).

As for mtDNA content after nuclear transfer, homoplasmy and heteroplasmy conditions have been reported to date. Homoplasmy for the donor cell mtDNA in intraspecific nuclear transfer (SMITH *et al.* 2000) and interspecific nuclear transfer (CHEN *et al.* 2002); homoplasmy for the recipient oocyte mtDNA (DO *et al.* 2001; DO *et al.* 2002; EVANS *et al.* 1999; HIENDLEDER *et al.* 2003; MEIRELLES *et al.* 2001; TAKEDA *et al.* 2003; TAKEDA *et al.* 1999; TAMASSIA *et al.* 2004); and heteroplasmy from both sources of mtDNA, recipient oocytes and donor cells in different proportions (BURGSTALLER *et al.* 2007; DO *et al.* 2002; FERREIRA *et al.* 2007; HAN *et al.* 2004; HIENDLEDER *et al.* 1999; LLOYD *et al.* 2006; MA *et al.* 2008; MEIRELLES *et al.* 2001; SANSINEMA *et al.* 2011; ST. JOHN and SCHATTEN 2004; STEINBORN *et al.* 2002; STEINBORN *et al.* 2000; TAKEDA *et al.* 2003; TAKEDA *et al.* 2008; TAKEDA *et al.* 2006). Most of the heteroplasmic results showed mild heteroplasmy, ranging between 0.1 and 13.9 % (BURGSTALLER *et al.* 2007; FERREIRA *et al.* 2007; HIENDLEDER *et al.* 2003; LLOYD *et al.* 2006; MA *et al.* 2008; MEIRELLES *et al.*

2001; STEINBORN *et al.* 2002; STEINBORN *et al.* 2000; TAKEDA *et al.* 2008; TAKEDA *et al.* 2006) whereas others showed higher levels, ranging between 17 to 57.43% (BURGSTALLER *et al.* 2007; HIENDLEDER *et al.* 1999; TAKEDA *et al.* 2003; TAKEDA *et al.* 2008; TAKEDA *et al.* 2006).

Homoplasmy for the recipient oocyte's mtDNA has been explained by the possible destruction of donor mtDNA by mechanism of elimination similar to those operating to eliminate sperm mtDNA in intraspecific fertilization (EVANS *et al.* 1999). It has also been suggested that mature mitochondria from donor cell may be destroyed by morphological transformation of these organelles during early embryonic development (TAKEDA *et al.* 1999).

Higher levels of heteroplasmy observed in nuclear transfer experiments using early embryonic cells have been explained by a replicative advantage of blastomere mtDNA (HIENDLEDER *et al.* 1999). Others have suggested that the levels of heteroplasmy in nuclear transfer clones depends of the quantity of donor mtDNA introduced at the moment of embryo reconstruction (TAKEDA *et al.* 2003) or that the transplanted donor nucleus enables the preferential replication of its own mtDNA rather than the oocyte's foreign mtDNA (MORAES *et al.* 1999; SMITH and ALCIVAR 1993). In interspecific cloning, the complete substitution of mtDNA from rabbit oocyte for mtDNA of giant panda somatic cells is explained by genetic divergence of the species in which nuclei from giant panda donor cells

could support biogenesis of mitochondria from panda cells but not from rabbit oocytes (CHEN *et al.* 2002).

Due to the contrasting findings described above, it has been suggested that the degree of donor mtDNA transmission in SCNT experiments appears to be random and there is no explanation to the diverse segregation patterns (LLOYD *et al.* 2006).

Recently, two different approaches of heteroplasmy experiments made by the same group obtained different results. Buffalo ooplasm was transferred to bovine zygotes and the heteroplasmy was detected at the blastocyst stage (9.3%) with a subsequent drift to homoplasmy (from recipient zygotes) in offspring (CHIARATTI *et al.* 2010). In another experiment, damaged bovine embryos by treatment with ethidium bromide were rescued by ooplasm transfer from normal oocytes and produced heteroplasmic calves (CHIARATTI *et al.* 2011).

The aim of this project was to determine if mtDNA segregation in cattle clones derived by SCNT varies during embryonic, fetal and post-natal life.

## 2.3. Material and Methods

### *2.3.1. Somatic cell nuclear transfer*

The establishment of fibroblast cell lines, production of host cytoplasts, reconstruction of embryos by nuclear transfer, synchronization of recipient heifers and embryo transfer followed the protocols described in previous experiments carried on in our laboratory, with slight modifications (BORDIGNON *et al.* 2003).

Cultured fibroblasts obtained from a male bovine fetus, extracted at 50 days of gestation, were used as donor cells. The fetus was conceived by artificially inseminating (AI) to a Holstein heifer carrying the mtDNA mutation comprised of an insertion of 66 bp in the D-loop (3 repetitions of 22 bp). This mutation was used as a tracer to measure the amount of donor cell mtDNA introduced at SCNT during the growth and development of the clones. Recipient oocytes were collected from Holstein ovaries obtained from the local slaughterhouse, which carried the wild-type (*Bos taurus*) mtDNA.

### *2.3.2. Recovery of embryos, fetuses and tissues*

All animal experimental protocols were approved by the Comité d'éthique de l'utilisation des animaux, Faculté de médicine vétérinaire, Université de Montréal; in

accordance with regulations of the Canadian Council for Animal Care. Thirty SCNT clones of different ages were produced: 17 day old embryos (n=17); 40 day old fetuses (n=3); 60 day old fetuses (n=3); one 240 day old fetus; and three adults, which were first biopsied at 18 months of age and sampled postmortem at 24 months of age. The proportion of heteroplasmy from donor cells mtDNA was calculated in whole embryos. The proportion of heteroplasmy was quantified in each tissue collected from fetuses and adults.

### *2.3.3. Quantification of mutant mtDNA*

Genomic DNA (gDNA) was extracted with the DNeasy tissue kit (Quiagen, Mississauga, Ontario, Canada) and quantified by spectrophotometry (GeneQuant *pro*, Biochrom, Cambridge, UK). To establish a quantitative mtDNA assay, both mutant (MT) and wild-type (WT) gDNA were diluted to obtain a seven-point standard curve (SC) developed from a mixture of these two types of mtDNA with increasing concentrations of MT mtDNA (0.1 %, 0.2%, 0.4%, 0.8%, 1.6%, 3.2% and 6.4%). A 5 µl aliquot from each dilution point in the standard curve were used as templates (1 ng/µl) for the nested PCR, together with the unknown experimental samples.

The gDNA from embryos, fetuses and adult clone tissues was extracted and quantified as described above. An aliquot of 50 ng of gDNA from each sample was used as template. PCR amplification was performed in 50 µl of reaction volume containing 1X

PCR buffer (Amersham, Baie d'Urfé, Québec, Canada), 0.2 mM dNTP (Promega, Nepean, Ontario, Canada), 1 pmol of each primer (QIAGEN), and 5 units of Taq polymerase (Amersham). The reaction mixture was incubated in a thermal cycler (Touchgene Gradient, Techne, Duxford, Cambridge, UK) under the following conditions: 94 $^{o}$C during 2 min for first denaturation; 40 denaturation cycles, annealing, and extension (94 $^{o}$C for 30 sec, 56 $^{o}$C for 30 sec, and extension for 30 sec at 72 $^{o}$C), and 72 $^{o}$C during 5 min for final extension. Primers are described in table 2.

The products derived from the first PCR were purified by using a QIAquick PCR Purification Kit Protocol (QUIAGEN), quantified and diluted to attain 0.2 ng/µl concentrations. Aliquots (5 µl) from each dilution were used as templates (1 ng/µl) and a standard curve, along with the set of unknown samples, was run for each PCR as described above. The products of the second (nested) PCR were run on a 1.5% agarose gel, stained with ethidium bromide and analyzed by densitometry (Alpha-Imager $^{TM}$ 2200, Alpha Innotech, San Leandro, CA, USA). A linear regression was estimated with an $R^2 = 0.98$ as the minimum coefficient of determination to calculate the proportions for the unknown samples. Three repetitions were made at least for each gel and the lowest threshold of detected heteroplasmy was 0.01%.

### *2.3.4. Statistical analysis*

Distinct mixed linear models were used for each analysis: (1) a mixed model in which period of life (pre-natal or post-natal) was considered a fixed factor and animal identification, nested within period, as a random variable, to take repeated measurements within each clone into account; (2) a mixed linear model in which stage was considered a fixed factor and animal identification, nested within stage as a random variable, to take repeated measurements within each clone into account; (3) a mixed linear model in which age was considered a fixed factor and animal identification, nested within age as a random variable to take repeated measurements within each clone into account; (4) a repeated measures linear model in which tissue was considered a repeated factor; (5) a repeated measures linear model where type (placenta or fetus) was considered a repeated factor, and (6) a repeated measures linear model where mitosis was considered a repeated factor.

## 2.4. Results

In these experiments we analyzed 30 bovine SCNT clones at different periods after oocyte reconstruction: 17 embryos at 17 days, 3 fetuses at 40 days, 3 fetus at 60 days, 1 fetus at 240 days and 3 adults at 810 and 990 days post SCNT. Three types of tissues (semen, skin and white blood cells) were sampled at 810 days and 12 types of tissues (brain, cerebellum, heart, intestine, kidney, liver, lung, muscle, spleen, stomach, testicle and

tongue) at 990 days post SCNT. In most (95.2%) of the tissues analyzed (99/104 tissues) the mean heteroplasmy levels attained was 1.46%, which is close to neutral segregation of mtDNA (~ 0.9%) reported by Steinborn *et al*. (2002). However, the 5 types of tissues from a Day-40 fetus (fe-40-1) showed substantially higher levels of heteroplasmy (mean of 20.85%) and, therefore, was considered an outlier and excluded from the general statistical analysis (Table 1, Figure 9). Moreover, only the ovary from one 240 days old female fetus was found to be homoplasmic as to the recipient oocyte's mtDNA whereas the other tissues (10/11) collected from this female fetus were heteroplasmic (Table 1).

Previous studies have indicated that mtDNA segregation patterns change after birth (HIENDLEDER *et al.* 1999; ST. JOHN and SCHATTEN 2004; STEINBORN *et al.* 2002; TAKEDA *et al.* 2003; TAKEDA *et al.* 2008; TAKEDA *et al.* 2006). Therefore, we classified our samples into two periods: pre-natal period (including both embryos and fetuses) and post-natal period (including both the 810 and 990 days old adults). We did not find a significant difference ($p = 0.39$) in heteroplasmy levels between these two periods of life (Figure 1). When we analyzed the effect of age in more detail, we did not find significant differences ($p = 0.40$) in heteroplasmy (Figure 2).

Cellular proliferation and turnover is known to vary in different tissues and mtDNA segregation is directly correlated with mitotic divisions (TERMAN *et al.* 2010). Therefore, the effect of tissue type on mtDNA segregation was examined in tissues sampled from fetuses and adults. No significant differences were found in heteroplasmy levels among

fetal tissues ($p = 0.13$; Figure 3) or adult tissues ($p = 0.74$ Figure 4). To analyze the effects of mitotic activity we classified the tissues into mitotic (intestine, kidney, liver, lung, skin, spleen, stomach and testicle) and post-mitotic (brain, cerebellum, heart, muscle, semen, tongue and white blood cells). No significant differences ($p = 0.48$) in heteroplasmy were found between the mitotic and post-mitotic tissues (Figure 6). These results indicate that segregation of mutated mtDNA from SCNT tissues did not follow the rules of different segregation patterns of mtDNA between mitotic and post-mitotic tissues reported by other researchers (CORTOPASSI *et al.* 1992).

Previous studies have indicated that mtDNA segregation patterns may differ among embryonic and extra embryonic tissues (MEIRELLES *et al.* 2001). Therefore, we analyzed the mtDNA heteroplasmy levels in SCNT-derived fetal (brain, heart, liver and muscle) and placental (cotyledons and the umbilical cord) tissues. No significant differences ($p = 0.91$) were found in heteroplasmy between fetal and placental tissues (Figure 5).

Tissues coming from the 40-day-old fetus (Fe-40-1), which was considered an outlier, showed high levels of heteroplasmy in several tissues: 17.23% in brain, 21.54% in cotyledon, 10.77% in heart, 36.76% in liver and 17.94% in muscle. The mean heteroplasmic proportion for this fetus reached 20.85 % (Figure 7). Figure 8 is a typical agarose gel showing the nested PCR bands of standard curve (different percentages of wild and mutated mtDNA) and unknown samples from the outlier clone (Fe-40-1). The points of the linear regression were obtained from the values of densitometry of the different bands

of the standard curve, and they were used to calculate the percentage of heteroplasmy of unknown samples.

Figure 9 shows the mean mtDNA heteroplasmy levels of all SCNT clones produced in these experiments. The means of heteroplasmy in 17 embryos of 17 days (1.60%), 2 fetuses of 40 days (1.01%), 3 fetuses of 60 days (1.42%), 1 fetus of 240 days (1.29%), 3 adults of 810 days (1.78%) and 3 adults of 990 days (1.41%) were inside the values of neutral segregation. Only one clone of 40 days (Fe-40-1) presented higher levels of heteroplasmy (20.85%) and was considered an outlier.

**2.5. Discussion**

In SCNT experiments, recipient oocytes have been obtained from slaughterhouse ovaries (DO *et al.* 2002; FERREIRA *et al.* 2007; HAN *et al.* 2004; HIENDLEDER *et al.* 2004; HIENDLEDER *et al.* 2003; MEIRELLES *et al.* 2001; TAKEDA *et al.* 2003; TAKEDA *et al.* 2008; TAKEDA *et al.* 1999) and from superovulated females from defined origin (BURGSTALLER *et al.* 2007; EVANS *et al.* 1999; HIENDLEDER *et al.* 1999; ST. JOHN and SCHATTEN 2004). In SCNT experiments when the recipient oocytes are collected from slaughtered cows or from superovulated unrelated cows, the produced clones could not share a common cytoplasm and may differ by cytoplasmic genetic effects (HIENDLEDER *et al.* 1999). Different sources of recipient oocytes can increase the chance to produce divergent donor mtDNA segregation patterns in SCNT (BURGSTALLER *et al.* 2007).

In SCNT studies in which heteroplasmy was estimated in cloned embryos, fetuses and progeny, different types of donor cells have been used: blastomeres (FERREIRA *et al.* 2007; MEIRELLES *et al.* 2001; ST. JOHN and SCHATTEN 2004; STEINBORN *et al.* 1998; TAKEDA *et al.* 1999), fetal fibroblasts (BURGSTALLER *et al.* 2007; MA *et al.* 2008; STEINBORN *et al.* 2000; TAKEDA *et al.* 2003; TAKEDA *et al.* 2006), adult fibroblasts (FERREIRA *et al.* 2007; HAN *et al.* 2004), adult epithelial cells (EVANS *et al.* 1999; TAKEDA *et al.* 2003; TAKEDA *et al.* 2008), adult cumulus cells (DO *et al.* 2002; TAKEDA *et al.* 2003) and adult granulosa cells (HIENDLEDER *et al.* 2003; STEINBORN *et al.* 2002).

Mitochondria coming from blastomeres collected from morula are before mtDNA replication phase that occurs either at or after the blastocyst stage in cattle (SMITH *et al.* 2005). Probably the somatic cells used as donor cells are still programmed for transcription and replication of mtDNA. In embryos produced by SCNT, the persistence of nuclear-encoded mtDNA transcription and replication factors (from the donor cell) but lacking in embryos produced by in vitro fertilization, were observed. For that reason, after nuclear transfer the nuclear-mitochondrial interactions are not coordinated because the onset of mitochondrial replication is a postimplantation event in natural or in vitro fertilization (LLOYD *et al.* 2006).

For introducing donor cells into enucleated oocytes in SCNT heteroplasmy experiments, the electrofusion is the most frequently technique used (EVANS *et al.* 1999; FERREIRA *et al.* 2007; HAN *et al.* 2004; HIENDLEDER *et al.* 2004; HIENDLEDER *et al.* 1999;

HIENDLEDER *et al.* 2003; MA *et al.* 2008; MEIRELLES *et al.* 2001; TAKEDA *et al.* 2003; TAKEDA *et al.* 2008; TAKEDA *et al.* 1999) and microinjection is used less frequently (DO *et al.* 2002; TAKEDA *et al.* 2006). Meirelles and Smith (1998) showed that segregation patterns of mitochondria were different according to the place where the mitochondria were introduced, in the periphery or around the nucleus of murine zygotes, with preferential replication of perinuclear mitochondria (MEIRELLES and SMITH 1998). This phenomenon could affect the heteroplasmy outcomes mainly when the technique of microinjection is utilized. After intraspecies fusion of eukaryotic cells the mitochondrial networks of the two cells fuse and the molecular contents of these networks are exchanged (YOON *et al.* 2007).

The mitochondria's shape changes through frequent fusion, fission and the mitochondria move throughout the cell. These dynamics are essential for the life and death of cells because they are involved in maintenance of cellular homeostasis and apoptosis (OTERA and MIHARA 2011). The mitochondrial morphology is controlled by a balance between mitochondrial fusion and fission. The physiological importance of mitochondrial fission is less understood than mitochondrial fusion (ISHIHARA *et al.* 2009). The mitochondrial fusion is critical to transfer mtDNA within and between the mitochondrial networks (YOON *et al.* 2007). Because fusion allows cooperation between mitochondria, it has a protective effects on the mitochondrial population and is essential for embryonic development (CHEN *et al.* 2003). Because the mitochondria cannot be generated *de novo*, the fission of pre-existing organelles is indispensable for the generation of new

mitochondria (AMIRI and HOLLENBECK 2008). MtDNA molecules seem to be selected at random for replication and double in every cell cycle (ALBERTS *et al.* 2002).

The mtDNA is packaged in nucleoids, composed of one to ten copies of mtDNA associated with proteins. It is not known whether the mtDNA copy number per nucleoid is constant or vary among different cells. Different forms of mtDNA can exist inside the cells and tissues, this condition known as heteroplasmy. Wild type and mutant mtDNA are distributed throughout the mitochondrial network. The nucleoids carrying different mtDNAs (heterologous) do not appear to exchange genetic material and remain genetically autonomous (GILKERSON 2009). Jacobs *et al*. (2000) proposed that heteroplasmic state of the cell depends on how the wild and mutant mtDNA are organized in cell's nucleoids (faithful nucleoid model). Two different situations were proposed. First, when the two different mtDNA are maintained in different nucleoid population, the cell's overall heteroplasmy will be fluid, subject to many variables like selective pressure and replicative advantage. Second, when the two mtDNAs are maintained within the same nucleoid population, the cell's overall heteroplasmy will remain relatively static (JACOBS *et al.* 2000).

The ability to select against deleterious mutations in mtDNA has been reported. Somatic cells package multiple mtDNA genomes in a single nucleoid complex. These cells have a more complex organization of the nucleoids than do oocytes. This fundamental

difference suggests that oocytes may be better able to detect and remove defective mtDNA genomes than somatic cells, possibly due in part to the simpler organization of the mtDNA in smaller nucleoids (BOGENHAGEN 2010).

If there is a condition of heteroplasmy in tissues, the proportion of wild-type and mutant mtDNA will change in the successive mitotic divisions among the daughter cells. If the proliferation continues, the mtDNA populations in daughter cells drift towards homoplasmy for wild or mutant mtDNA (segregation). This condition is not applicable in postmitotic tissues, where cells are not dividing. Probably, there are tissue-specific mechanisms favoring accumulation of particular mtDNA (HOFHAUS *et al.* 2003).

The quantity of mtDNA copies by cell varies depending of the stage of life. Each of the cell from the embryo's inner cell mass contains approximately 1,000 copies of mtDNA (EVANS *et al.* 1999), in ovine fetal fibroblasts were calculated between 292 to 753 copies of mtDNA per cell (BURGSTALLER *et al.* 2007) and adult somatic cells have between $10^3$ and $10^4$ copies of mtDNA, with 2–10 copies per organelle (SPIKINGS *et al.* 2006). These differences in quantity and quality of mitochondria among embryonic, fetal and adult cells could affect the fate of mtDNA donor cells when they are used in cell nuclear transfer experiments.

In our bovine SCNT experiments the main outcome was the neutral segregation of mtDNA, levels of heteroplasmy remaining similar to the quantity introduced at the moment of reconstruction of oocyte (~ 0.9%), reported by Steinborn *et al*. (2002). There were not significant differences in heteroplasmy between the pre-natal and post-natal periods of life, between fetal and adult clones, among the different tissues of fetuses, between the fetal and placental tissues of fetuses, among the different tissues of adults, and between mitotic and undividing tissues of fetal and adult clones. The heteroplasmy observed in adult clones did not affect their development because they were healthy and could attain adulthood. Also the observed levels of heteroplasmy were transmitted to the semen (Described in Steinborn *et al*. 2000). The levels of heteroplasmy in the different tissues range between 0.00 and 4.51% with a mean of 1.46%. Only one fetus of 40 days showed higher levels of heteroplasmy outside of neutral segregation (20.85%) and was considered an outlier.

The results obtained in our SCNT experiments could be explained by the faithful nucleoid model (JACOBS *et al.* 2000). In the first case, if the electrofusion produced the fusion of the two different sorts of mitochondria and the two mtDNAs (donor and recipient cells) were maintained within the same nucleoid population, the levels of heteroplasmy remained relatively static and produced the most neutral segregation results observed in these experiments. In the second case, if the electrofusion produced the fusion of the cells but was not able to produce the fusion of mitochondria, the two mtDNAs were maintained in different nucleoid population, the heteroplasmy produced was very fluid and affected by

many variables, the segregation was stochastic and produced the elevated levels of heteroplasmy observed in the outlier clone.

Probably, the introduction of somatic mitochondrial nucleoids (harboring foreign and mutated mtDNA) from donor cell (nucleoids with complex organization) into the recipient oocyte (mitochondrial nucleoids with simpler organization), were not detected and were not removed by the autophagic cell mechanisms of oocyte, and produced the persistence of the foreign somatic mtDNA in the cloned progeny observed in our experiments (similarly as described by Bogenhagen, 2010).

In our experiments, we did not observe the effect of collection of oocytes from slaughtered cows that reportedly can produce divergent donor mtDNA segregation patterns in SCNT (BURGSTALLER *et al.* 2007; HIENDLEDER *et al.* 1999). We did not observe differences in heteroplasmy between mitotic and post-mitotic tissues described by Hofhaus *et al*. 2003. Neither did we observe differences in heteroplasmy between fetal and placental tissues. We did not observe the tissue specific mtDNA segregation observed in karyoplast transplantation (SMITH and ALCIVAR 1993), cytoplast transplantation (TAKEDA *et al.* 2000) and SCNT experiments (TAKEDA *et al.* 2006). Most tissues from fetus and all tissues from adult clones presented neutral segregation of mtDNA with no differences among the tissues.

In our SCNT experiments, which used fetal fibroblast as donor cells, the results were quite different to previous experiments that used blastomeres as donor cells (BNT) harboring the same mutation in mtDNA (insertion of 66 bp). We did not observe the reported complete segregation to homoplasmy in both donor- and host-derived haplotypes in BNT experiments (SMITH *et al.* 2000). On the other hand, we observed a tendency to the neutral segregation of mtDNA (STEINBORN *et al.* 2002) as it was observed in almost all of the tissues collected from all the clones.

In summary, we conclude that neutral segregation of mtDNA was the main outcome in our SCNT experiments. This stable segregation suggests that after electrofusion the mitochondria from the two cells rapidly fuse and the two mtDNAs from both cells are maintained within the same nucleoid population and the cell's overall heteroplasmy remains relatively static. Total number of organelle DNA molecules doubled in every cell cycle and the mitochondria were divided by fission and shared in an equitable way in the successive mitotic divisions, producing the stable mitochondrial segregation observed in clones from the early embryo to adulthood. Further research must be done to elucidate whether the segregation of donor cell mtDNA in SCNT experiments could be a predictable or a random phenomenon.

## 2.6. Acknowledgements

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**Table 1. Heteroplasmy in embryos, fetus and adult bovine somatic cell nuclear transfer clones**

| Clone | Stage | Age | Br | Co | Cb | Ht | In | Kd | Lg | Lv | Mu | Ov | Se | Sk | Sp | St | Ta | Te | Th | To | UC | WBC | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Em-17-17 | Embryo | 17 | | | | | | | | | | | | | | | | | | | | | 1.60 |
| Fe-40-1* | Fetus | 40 | 17.23 | 21.54 | | 10.77 | | | | 36.76 | 17.94 | | | | | | | | | | | | 20.85 |
| Fe-40-2 | Fetus | 40 | 1.36 | 0.82 | | 1.12 | | | | 0.90 | 1.42 | | | | | | | | | | | | 1.12 |
| Fe-40-3 | Fetus | 40 | 0.57 | 1.39 | | 0.96 | | | | 0.68 | 0.91 | | | | | | | | | | | | 0.91 |
| Fe-60-1 | Fetus | 60 | 0.95 | 0.78 | | 0.32 | | 1.35 | | 1.23 | 0.87 | | | 0.90 | | 1.68 | 1.73 | | | 1.60 | 4.51 | | 1.45 |
| Fe-60-2 | Fetus | 60 | 0.41 | 0.18 | | 0.14 | | 0.16 | 0.06 | 0.67 | 1.50 | | | | | | | | | | | | 0.45 |
| Fe-60-3 | Fetus | 60 | 1.37 | 1.39 | | 1.16 | 1.86 | 1.45 | 2.07 | 1.79 | 1.20 | | | 1.20 | | 1.69 | | 1.57 | 1.39 | 1.15 | 0.97 | | 1.50 |
| Fe-240-1 | Fetus | 240 | 0.28 | 0.02 | | 0.50 | 4.52 | 0.10 | 0.12 | 0.25 | 0.14 | 0.00 | | | 6.74 | | | | | 1.53 | | | 1.29 |
| Ad-18-A | Adult | 810 | | | | | | | | | | | 1.56 | 0.79 | | | | | | | | 1.11 | 1.18 |
| Ad-18-B | Adult | 810 | | | | | | | | | | | 2.06 | 2.28 | | | | | | | | 3.55 | 2.59 |
| Ad-18-C | Adult | 810 | | | | | | | | | | | 1.62 | 1.50 | | | | | | | | 1.57 | 1.57 |
| Ad-24-A | Adult | 990 | 0.74 | | 1.19 | 1.00 | 1.17 | 1.69 | 1.67 | 1.82 | 1.27 | | | | 1.10 | 1.14 | | 2.71 | | 0.57 | | | 1.34 |
| Ad-24-B | Adult | 990 | 1.10 | | 1.36 | 1.04 | 2.83 | 0.98 | 2.35 | 1.02 | 1.58 | | | | 2.46 | 0.71 | | 0.88 | | 1.69 | | | 1.52 |
| Ad-24-C | Adult | 990 | 0.52 | | 1.42 | 0.91 | 1.38 | 1.39 | 1.11 | 0.85 | 1.41 | | | | 0.71 | 1.53 | | 1.38 | | 2.56 | | | 1.26 |

Analyzed tissues: Brain (Br), cotyledon (Co), cerebellum (Cb), heart (Ht), intestine (In), kidney (Kd), lung (Lg), liver (Lv), muscle (Mu), ovary (Ov), semen (Se), skin (Sk), spleen (Sp), stomach (St), tail (Ta), testicle (Te), thymus (Th), tongue (To), umbilical cord (UC) and white blood cells (WBC).
The total heteroplasmy was analyzed in each embryo (no defined organs).
Empty spaces mean not analyzed tissues
The age is considered since the SCNT reconstruction of embryos
Fetus 40-1*. This fetus was considered like an outlier and was not included in the total statistical analysis.

**Table 2. Description of primers utilized in amplification of mutated region in the displacement loop of bovine mtDNA**

| Primer name | PCR | Function | Length (bp) | Tm | Sequence of primers (5' to 3') | mtDNA position | Amplicon (bp) |
|---|---|---|---|---|---|---|---|
| 15913BOV-F | First PCR | Forward | 22 | 63 | CATAACACGCCCATACACAGAC | 15913 | 283pb |
| 16176BOV-R | First PCR | Reward | 20 | 63 | TAGCGGGTTGCTGGTTTCAC | 16176 | |
| 15938n-F | Nested | Forward | 21 | 57 | AGAATGAATTACCTACGCAAG | 15938 | 222pb |
| 16140n-R | Nested | Reward | 20 | 58 | GCTCGTGATCTAATGGTAAG | 16140 | |

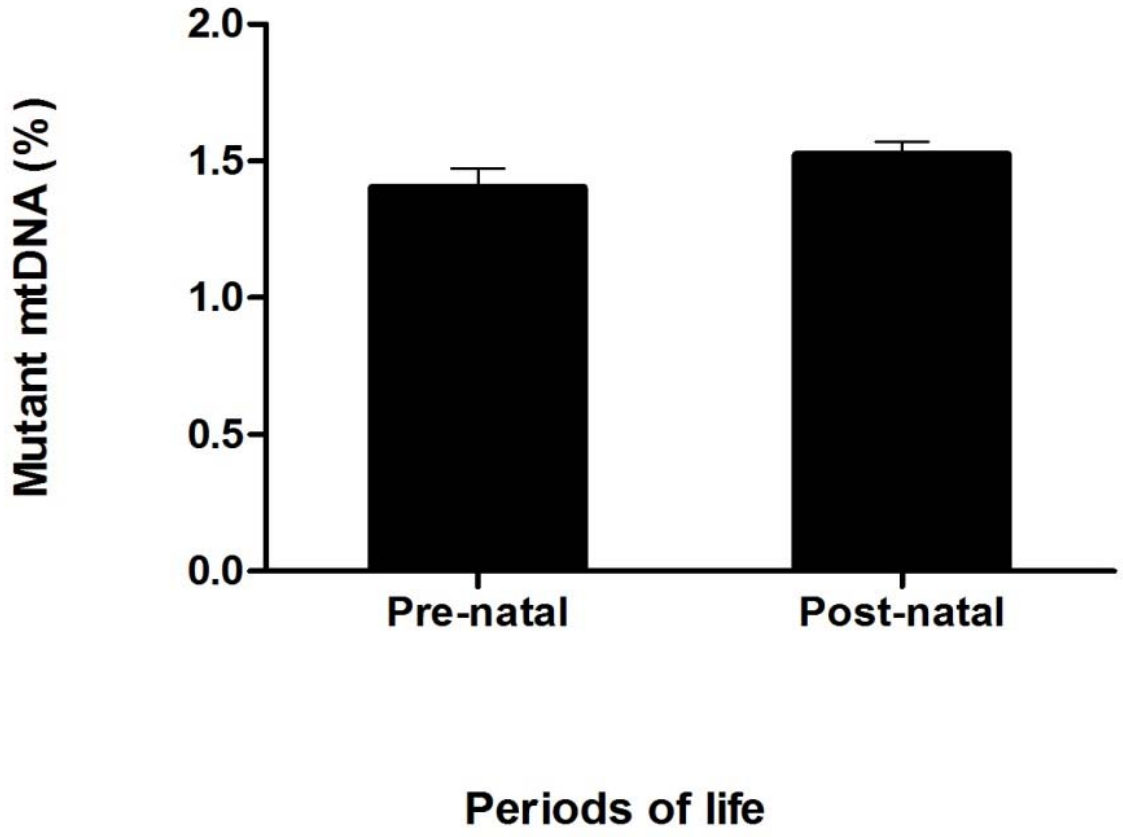



Figure 1. Presence of mtDNA from the donor cell in bovine SCNT clones of different periods of life. Means and standard errors of the mean are shown.

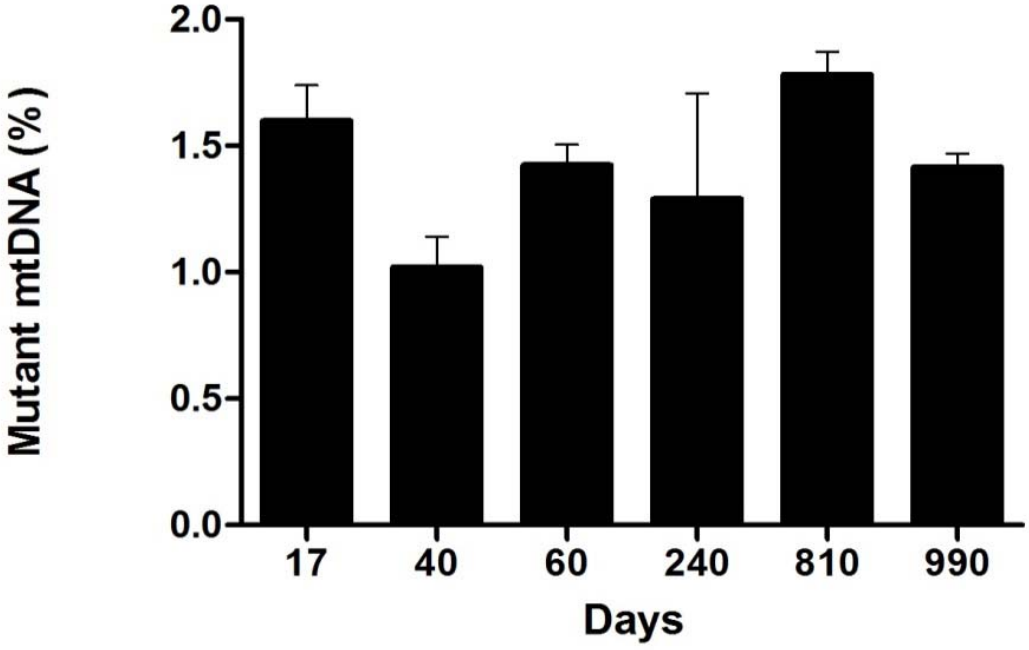



Figure 2. Presence of mtDNA from the donor cell in bovine SCNT clones of different ages. Means and standard errors of the mean are shown.

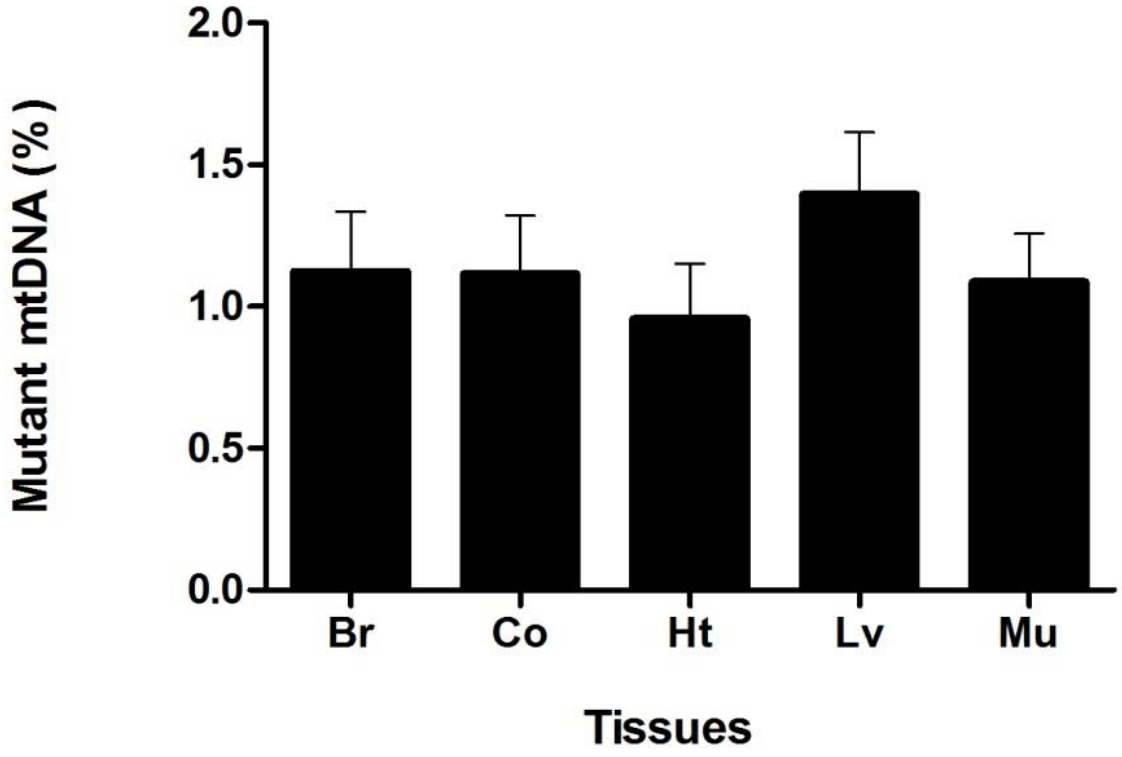



Figure 3. Presence of mtDNA from the donor cell in different tissues of SCNT-derived fetus. Means and standard errors of the mean are shown.

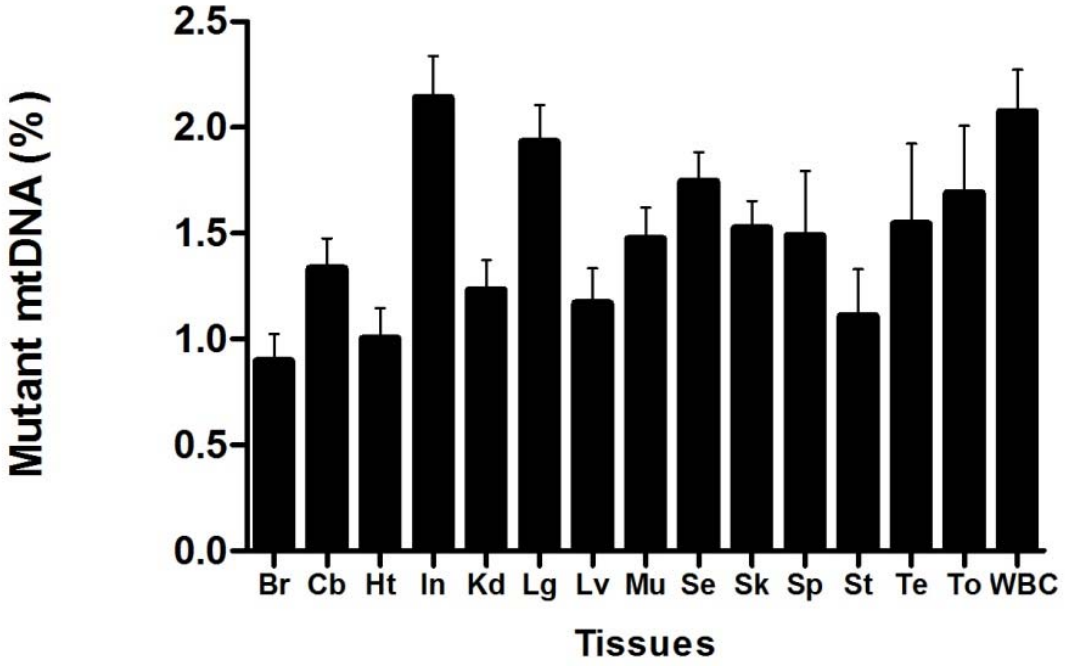



Figure 4. Presence of mtDNA from the donor cell in different tissues of SCNT-derived adult tissues. Brain (Br), cerebellum (Cb), heart (Ht), intestine (In), kidney (Kd), lung (Lg), liver (Lv), muscle (Mu), semen (Se), skin (Sk), spleen (Sp), stomach (St), testicle (Te), tongue (To) and white blood cells (WBC). Means and standard errors of the mean are shown.

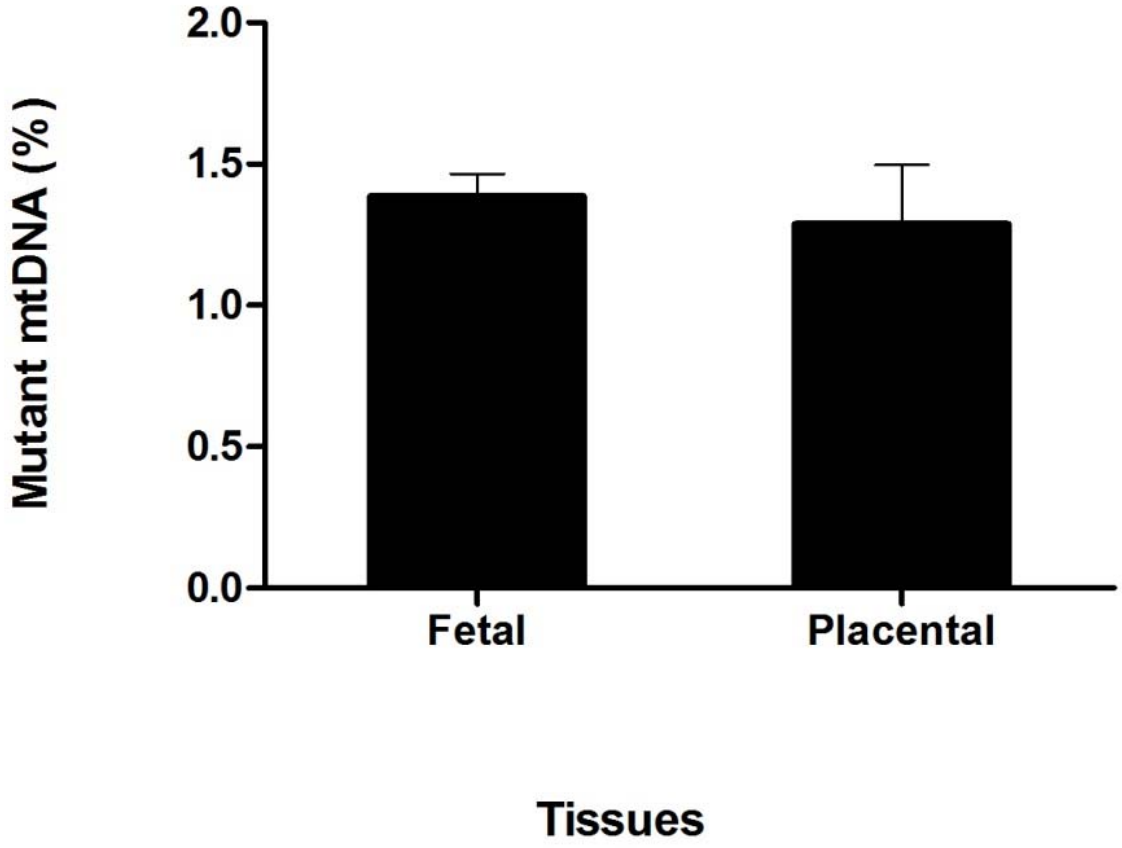



Figure 5. Presence of mtDNA from the donor cell in fetal and placental tissues of bovine SCNT clones. Means and standard errors of the mean are shown.

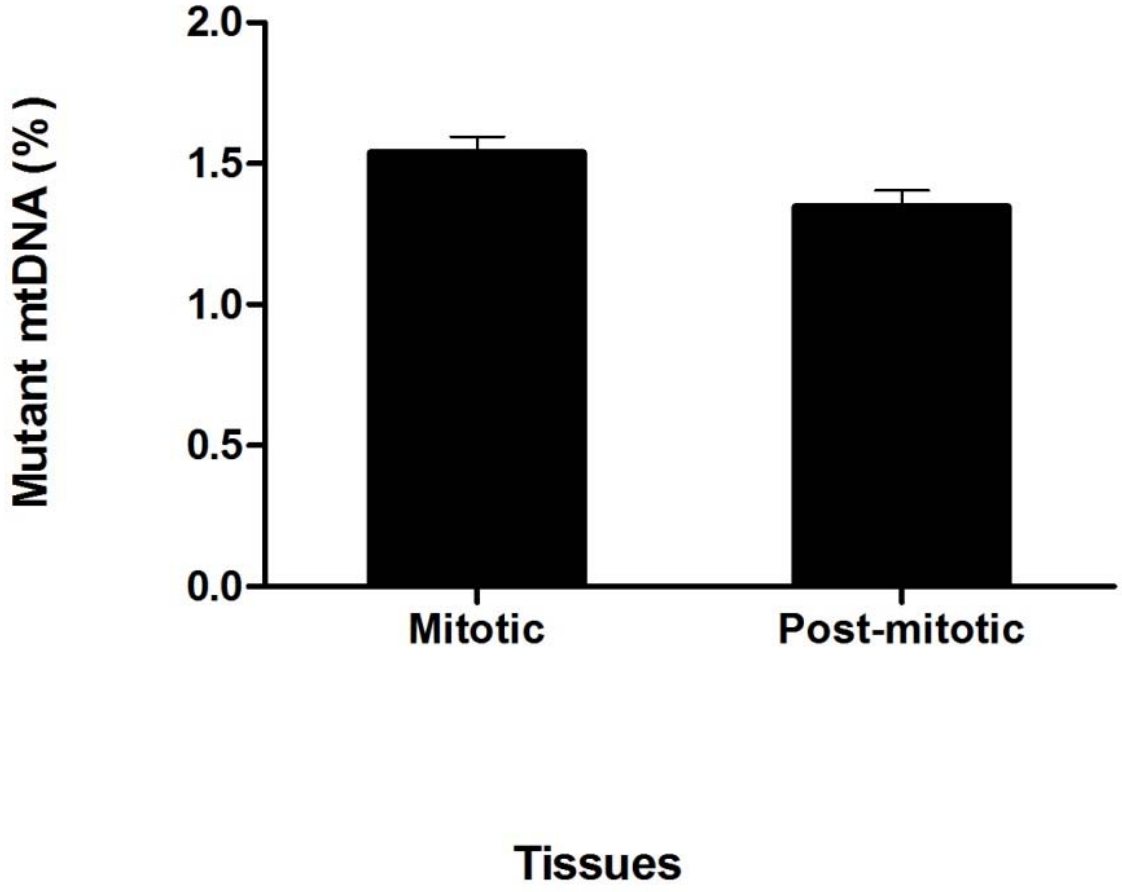



Figure 6. Presence of mtDNA from the donor cell in mitotic and post-mitotic tissues of bovine SCNT clones. Means and standard errors of the mean are shown.

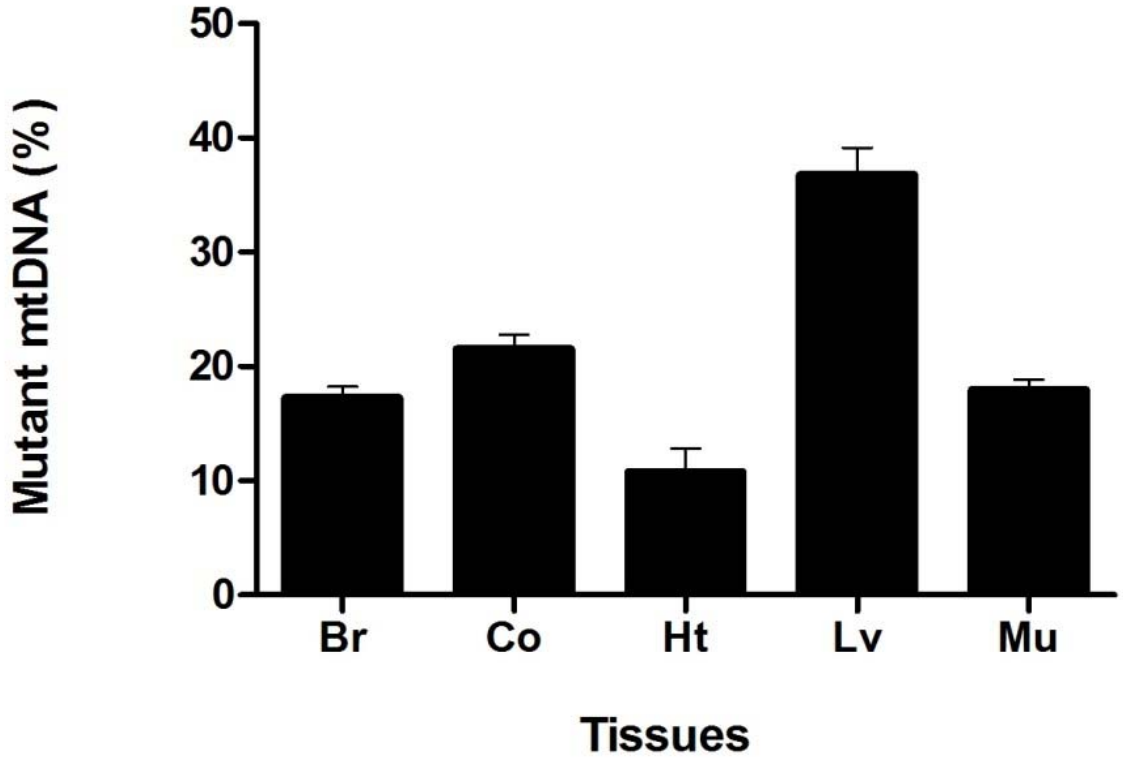
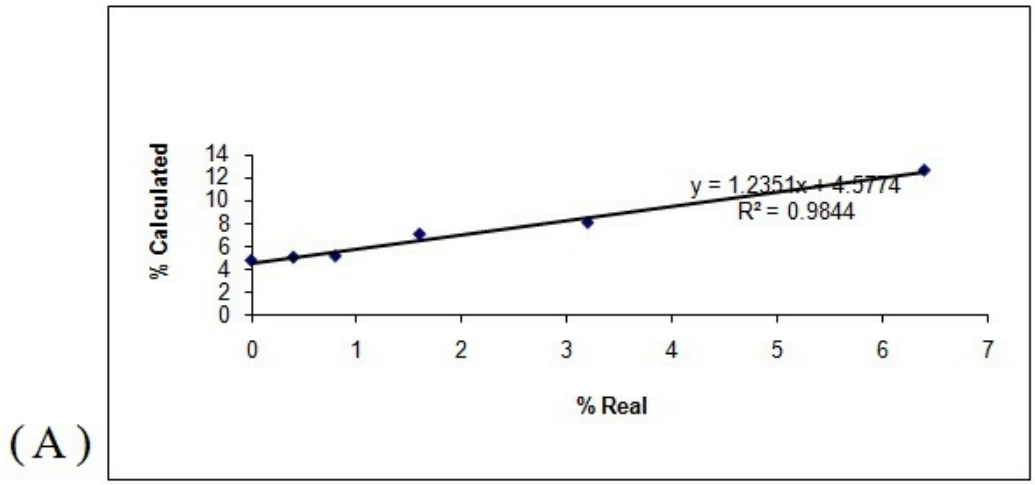



Figure 7. Presence of mtDNA from the donor cell in tissues of fetus Fe-40-1* (40-day-old fetus). Means and standard errors of the mean are shown. Brain (Br), cotyledon (Co), heart (Ht), liver (Lv) and muscle (Mu).



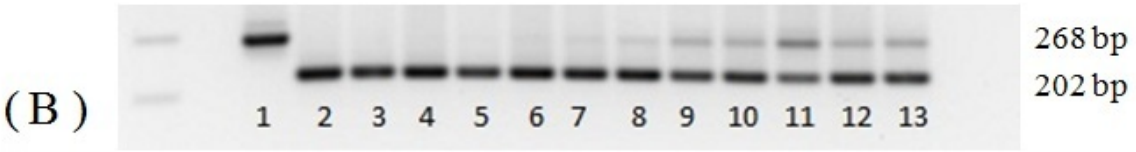




Figure 8. Standard curve and agarose gel of five analyzed tissues from fetus 40-1* (outlier). (A) Typical linear regression of standard curve utilized to calculate the unknown samples. (B) Electrophoretic bands of nested PCR products in agarose gel: 1 = 100% mutant mtDNA (Mt mtDNA), 2 = 100% wild mtDNA (Wd mtDNA), 3 = 0.2% Mt mtDNA, 4 = 0.4% Mt mtDNA, 5 = 0.8% Mt mtDNA, 6 = 1.6% Mt mtDNA, 7 = 3.2% Mt mtDNA, 8 = 6.4% Mt mtDNA, 9 = Brain, 10 = Heart, 11 = Liver, 12 = Muscle, 13 = Cotyledon.

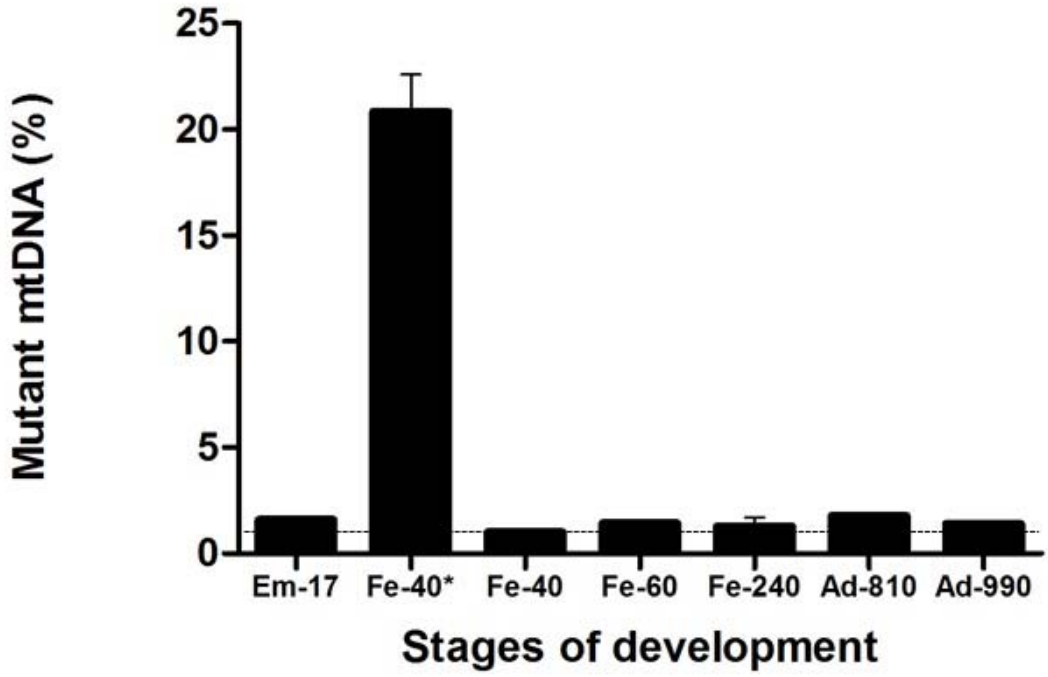



Figure 9. Different stages of development from all the SCNT clones produced in these experiments. Means and standard errors of the mean (SEM) are shown from the whole embryos and all the tissues mixed from fetuses and adult clones. 17 embryos of 17 days (Em-17), 1 fetus of 40 days (Fe-40*, outlier), 2 fetuses of 40 days (Fe-40), 3 fetuses of 60 days (Fe-60), 1 fetus of 240 days (Fe-240), 3 adults of 810 days (Ad-810) and 3 adults of 990 days (Ad-990). The transversal dotted line shows the expected percentage of mtDNA from the donor cell in reconstructed oocytes at the moment of the reconstruction (~ 0.9%, according with Steinborn *et al*. 2002).

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# Chapter III

## Somatic and germ cell changes in mitochondrial DNA during aging, meiosis, and early development in female mice

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## 3.1. Abstract

Female fertility is known to decrease with aging in most mammals but there is no conclusive relationship between this process and the mitochondria of germinal tissues. The aim of this project was to elucidate if the decline in fertility in aging female mice is related to changes in mtDNA from germinal tissues (mtDNA copy numbers, mtDNA deletions and mitochondrial gene transcripts). The effects of aging in somatic tissues (brain, liver muscle and cumulus) were also analyzed. Females of different age groups (0-8, 8-16 and 16-24 months) were synchronized and sacrificed to obtain oocytes at germinal vesicle and metaphase-II stages both in vivo and vitro. 2-cell and blastocyst stage embryos in vivo and in vitro were also analyzed. Assessments were made of mtDNA total copy numbers, percentage of mtDNA common deletion and the expression of three genes: Ndufs4, Mt-nd2 and Mt-nd4. As expected female fertility decreased with age and aging affected the mtDNA in somatic tissues. However, female aging did not affect the mtDNA in cumulus cells and oocytes. Although the culture in vitro did not affect the mtDNA in most germinal tissues, mtDNA deletions increased during the resumption of meiosis and disappeared during early embryo development. Since we did not find effects of age in most mitochondrial parameters analyzed in oocytes and embryos, it was concluded that mtDNA common deletion in germ cells is mostly related to meiotic and developmental status rather than the process of aging.

**Key word**: Meiosis, early embryo development, aging, culture in vitro, total mtDNA, mtDNA common deletion, Ndufs4, Mt-nd2, Mt-nd4

## 3.2. Introduction

In mammalian cells, energy is produced through glycolysis and mitochondrial OXPHOS (WALLACE and FAN 2010). When diets are transformed into energy in the oxidative phosphorylation, reactive oxygen species (ROS) are generated as toxic by-products (WALLACE 2005). Contrary to popular belief, aerobic and anaerobic respirations are not mutually exclusive and, at any one time, both contribute to the energy requirement by the mammalian cell. There remains active discussion as to whether the oocyte and developing embryo rely on aerobic or anaerobic respiration prior to implantation (WILDING *et al.* 2009).

Complex I (NADH dehydrogenase) is the first and the biggest complex involved in OXPHOS and is the major entry point for electrons to get into the electron transport chain. It is embedded in the inner membrane of mitochondria, and is capable of dehydrogenating NADH. The mammalian complex I consists of about 46 subunits, and 7 of them are encoded by the mitochondrial genes (Mt-nd1, Mt-nd2, Mt-nd3, Mt-nd4L, Mt-nd4, Mt-nd5 and Mt-nd6) (BAI *et al.* 2005; TRIEPELS *et al.* 2001; WALLACE 2005). Ndufs4 is a nuclear encoded-protein (18 kDa) of the complex I with dual role: Regulation of complex I activity in response to the energetic status of the cell and as structural pillar in the complex assembly in the inner mitochondrial membrane (PAPA 2002; PAPA *et al.* 2004;

PETRUZZELLA and PAPA 2002). Mt-nd2 and Mt-nd4 are components of the hydrophobic protein fragment of the complex I (BAI *et al.* 2005).

The oocyte is the largest cell in the body and requires enough energy to support transcription and translation during oocyte maturation and any insufficiency in ATP availability could compromise the oocyte quality (EICHENLAUB-RITTER and PESCHKE 2002). The primordial oocyte is arrested at the dictyate state of the meiotic prophase I until the resumption of meiosis later in the reproductive live. In this way, some oocytes can stay in this resting phase for more than 40 years in humans and more than one year in mice. The primordial oocyte grows from 15-20 μm until 70-150 μm in fully grown oocyte. The number of mtDNA copies also increases about 100 times, suggesting an increase in oxygen mediated metabolism (DORLAND *et al.* 1998; HAMATANI *et al.* 2004). This increase in OXPHOS during oocyte maturation (VAN BLERKOM 2000; VAN BLERKOM *et al.* 1997) produce higher levels of ROS that can damage the mtDNA (TAYLOR and TURNBULL 2005). During aging, ROS can increase oxidative damage, which results in the eventual loss of cellular functions through a combination of energy insufficiency, signaling defects, apoptosis and replicative senescence (TAYLOR and TURNBULL 2005).

Aging is a multifactorial phenomenon characterized by a decline of the physiological performance in general, which can be explained by a diminished mitochondrial respiratory function due to oxidative damage to the mtDNA mainly by the generation of ROS. This damage is characterized by an accumulation of point mutations

and rearrangements (especially deletions) in most tissues at a rate much faster than for the nuclear genome (MANDAVILLI *et al.* 2002; TAYLOR and TURNBULL 2005; WALLACE 2005). There are mtDNA deletions ranging from 6 to 10 422 bp in size. The most frequent and best-characterized mtDNA deletion is 4977 bp in length (the common deletion) in humans (HOFHAUS *et al.* 2003), 4974 bp in mouse (TANHAUSER and LAIPIS 1995) and 4834 bp in rats (CASSANO *et al.* 2004). This common deletion is considered to be a marker for mutations in the mitochondrial genome (HOFHAUS *et al.* 2003).

The presence of this common deletion has been identified in somatic tissues from animals and humans (CORTOPASSI and ARNHEIM 1990; TANHAUSER and LAIPIS 1995). Some experiments have demonstrated the correlation between the presence of this deletion and aging in a variety of somatic tissues (COOPER *et al.* 1992; CORRAL-DEBRINSKI *et al.* 1992a; CORRAL-DEBRINSKI *et al.* 1992b; CORTOPASSI and ARNHEIM 1990; CORTOPASSI *et al.* 1992; LEE *et al.* 1994; LINNANE *et al.* 1990; SEIFER *et al.* 2002; SIMONETTI *et al.* 1992; SOONG *et al.* 1992) Some studies have identified the common deletion in oocytes and, in less proportion in early stage embryos (BRENNER *et al.* 1998). There is also a significant increase in the frequency of mtDNA deletion in oocytes that remain unfertilized (HSIEH *et al.* 2002), lower expression of mitochondrial genes in unfertilized oocytes and arrested embryos harboring mtDNA common deletion (HSIEH *et al.* 2004) and increased presence of common mtDNA deletion in oocytes and embryos after gonadotropins stimulation (GIBSON *et al.* 2005).

The decline of fertility with aging could be explained by depletion of the ovarian reserve, decreased circulating estradiol, a decrease in the ovarian follicles' response to estrogen, a decrease in the oocyte quality and the presence of chromosomal anomalies (LÉVY 2005; OTTINGER 2010). In human oocytes, the cytoplasmic aging has been associated with decline of mitochondrial functions, reduced transcription of genes involved in spindle assembly checkpoint and increased frequency of apoptosis (TATONE *et al.* 2006). Until now, a direct relationship between mitochondrial mutations in oocytes and reproductive success has not been demonstrated (MAY-PANLOUP *et al.* 2005).

The aim of this project was to elucidate whether the fall in female fertility during aging was related to changes in mtDNA of germinal tissues (mtDNA copy numbers, mtDNA common deletions and mitochondrial gene transcripts).

## 3.3. Material and methods

### *3.3.1. Source of mice*

All animal experimental protocols were approved by the Comité d'éthique de l'utilisation des animaux, Faculté de médicine vétérinaire, Université de Montréal; in accordance with regulations of the Canadian Council for Animal Care. Female C57BL/6 mice (Charles River Laboratory, St-Constant, Quebec, Canada) were housed up to a maximum of 2 years to form 3 age groups (periods): (1) young (1-8 months); (2) middle (8-

16 months) and (3) old (16-24 months), and used to obtain somatic tissues (brain, liver, muscle and granulosa cells), germinal vesicle (GV) oocytes, metaphase II (M-II) oocytes, 2-cell and blastocyst stage embryos. C57BL/6 males of confirmed fertility were used for natural fertilization and to obtain sperm for in vitro fertilization.

### *3.3.2. Media and collection of oocytes and embryos*

If not otherwise stated, all chemicals were purchased from Sigma Chemical (St. Louis, MO, USA). The media used in these experiments were KSOM, modified KSOM, and HEPES buffered-KSOM (H-KSOM), minimum essential medium (MEM) and modified MEM-α. KSOM is composed of 95 mM NaCl, 2.50 mM KCl, 0.35 mM $KH_2PO_4$, 0.20 mM $MgSO_4$, 10 mM Na-lactate, 0.2 mM D-glucose, 25 mM $NaHCO_3$, 0.2 mM Na pyruvate, 1.71 mM $CaCl_2$, 0.01 mM $Na_2EDTA$, 1 mM L-glutamine (Gibco, Invitrogen corporation, NY USA), 60 μg/ml penicillin-G, 50 μg/ml streptomycin sulfate and 4 mg/ml bovine serum albumin (BSA). In the modified KSOM, 2 % (v/v) basal medium Eagle (BME) essential aminoacids without L-glutamine and 1 % (v/v) MEM non-essential amino acids (Gibco) were added to the original KSOM. H-KSOM is composed of the same ingredients as original KSOM with final concentrations of $NaHCO_3$ at 4 mM and HEPES at 21 mM and phenol red (1% v/v). An aliquot of 100 ml of minimum essential medium is composed of 0.94 g MEM Eagles medium, 0.1 ml EDTA.4 Na (0.038 g/ml), 0.025 g sodium pyruvate, 0.22 g $NaHCO_3$, 0.1 ml penicillin (10000 IU/ml), 1.0 ml L-glutamine (29.2 mg/ml) (Gibco, Invitrogen corporation, NY USA) and 0.3 g BSA. MEM-

α (Invitrogen) was MEM supplemented with 5% fetal bovine serum, 25 mg/L sodium pyruvate, 75 mg/L penicillin G potassium, 50 mg/L streptomycin sulfate and 0.3 mg/L follicle stimulating hormone (modified from Eppig *et al*. 1994) (EPPIG *et al.* 1994).

To obtain oocytes at the GV stage, females from the 3 age groups were primed with 5 IU of pregnant mare serum gonadotropins (PMSG) (Folligon, Intervet, Ontario, Canada) followed (46-48 h later) by 5 IU of human chorionic gonadotropins (hCG) (Chorulon, Intervet, Ontario, Canada). Females were sacrificed 90 min after the injection of hCG and the ovaries were punctured with a fine needle to recover cumulus oocyte complexes (COCs) with intact cumulus layers. To obtain GV oocytes for analysis, cumulus cells were removed by pipeting in H-KSOM with hyalurodinase (20 IU/ml). After denudation, fully grown GV oocytes ($\geq$ 80 μm in diameter) were washed and stored at – 80 $^{\circ}$C in phosphate buffered saline (PBS) containing 0.1% (w/v) polyvinyl alcohol (PVA). Some GV oocytes were transferred to microdrops of modified MEM-α, and cultured for 14-16 h to enable meiotic resumption in vitro (TAKEUCHI *et al.* 2005). After maturation, M-II oocytes (with polar body extrusion) were washed and stored for further analysis as described above.

To obtain in vivo-derived M-II oocytes, superovulated females were sacrificed 14-16 h after hCG injection and oviducts with ova were dissected under sterile mineral oil and COCs were transferred to H-KSOM droplets (with hyalurodinase). Denudation of cumulus cells, washing and storage of M-II oocytes followed the protocol for GV oocyte collection

described before. It was not possible to obtain in vivo M-II oocytes from the old group of females (swollen oviducts with liquid and without oocytes).

In vitro fertilization (IVF) of in vivo derived M-II oocytes was performed with fresh semen according to Nagy *et al*. 2003 (NAGY *et al.* 2003). After IVF, zygotes were cultured for 1 (2-cell) and 4 (blastocyst) days to obtain in vitro-derived embryos for developmental analysis.

In vivo fertilized embryos were obtained from "copulation plugged" females on the second (2-cell) and fourth (blastocyst) days after caging with fertile males. The embryos were washed and stored in the same way described in the GV oocyte collection protocol.

### *3.3.3. Quantification of transcript abundance*

Messenger ribonucleic acid (mRNA) was extracted directly from the vials containing oocytes or embryos using magnetic beads following manufacturer's instructions (Dynabeads mRNA DIRECT Micro Kit, Dynal, Oslo, Norway) with modifications. Briefly, 5 pg rabbit α–globin mRNA was added to each sample and used as an external standard (WRENZYCKI *et al.* 1999). Each oocyte or embryo was lysed in 100 µl of lysis/binding buffer by gentle shaking for 10 min and 10 µl of pre-washed beads. After a second gentle shaking for 10 min, the beads were separated with a magnetic separator and the supernatant was used for the extraction of genomic DNA. The mRNA-Dynabeads complex was used

immediately for reverse transcription (RT) (37 °C for 60 min) into complementary DNA (cDNA) in a thermocycler (T3000, Biometra, Germany) using the Sensiscript reverse transcription kit (QIAGEN, Valencia, USA).

After the RT the beads were washed in 20 µl of a buffer containing: 100 µl dNTP (Amersham, Pittsburgh, USA), 200 µl ordinary buffers (Amersham), 0.2 µl SYBr green 10000 X (Roche, Mannheim, Germany), 100 µl BSA (10 mg/ml) and 1580 µl of water. At the end the beads were resuspended in 20 µl containing specific primer mix and premix of Lightcycler FastStart DNA Master SYBR Green I (Roche). The mix (20 µl) was amplified in a thermocycler by PCR using 2 cycles of 30 sec at 95 °C for denaturation, 30 sec at 54 °C for annealing and 30 sec at 72 °C for elongation. Amplified DNA of five genes (rabbit α-globin, Gapdh, Ndufs4, Mt-nd2 and Mt-nd4) were quantified using the Lightcycler 1.2. Instrument (Roche) with an amplification program of 40 cycles including 5 sec at 95 °C, 5 sec at 56 °C and 10 sec at 72 °C. All data of gene expression were presented as a ratio between the specific genes and the Gapdh.

### *3.3.4. Quantification of total and mutant mtDNA*

Total and mutant mtDNA from oocytes and embryos were quantified from the supernatant collected during the mRNA extraction protocol. Genomic DNA (gDNA) was extracted with a QIAamp DNA microkit following the protocol of isolation of genomic

DNA from small volumes of blood (QIAGEN). At the end, extracted DNA was eluted in 100 µl of distilled water and stored at 4 °C until further analysis. Quantification of total copies of wild mtDNA and copy numbers of mutant mtDNA was performed by PCR with 5 µl of gDNA and 15 µl of premix. The premix consisted of specific primer mix (see table 2), specific probe (UPL) (Roche) and Lightcycler TaqMan Master (Roche). A standard curve with 8 points was included in each reaction, i.e. a negative with total primers, a negative with deletion primers and 6 concentration points from 50 to 5,000,000 copies. The PCR program consisted of an initial step of activation of 95 °C for 600 sec followed by 40 cycles of 30 sec at 94 °C for denaturation, 30 sec at 60 °C for annealing-elongation and 1 sec at 72 °C for acquisition (single). Each reaction contained a gene specific primer and a fluorescently labeled TaqMan probe (Roche, UPL).

Somatic tissues were analyzed using gDNA extracted with Qiamp DNA Mini Kit (QIAGEN) and treated with RNase A (QIAGEN). For quantification of total and mutant mtDNA, gDNA was amplified with specific primers (Table 3) using a thermocycler Rotor Gene Q and Probe PCR Kit (QIAGEN). The total number of mitochondrial copies obtained was divided between the number of copies of Gapdh gene/2 (2 copies of Gapdh per cell) to obtain the number of copies by genome. The percentage of copies with deletion was obtained by dividing the number of mutant copies by the total mtDNA copies.

### *3.3.5. Statistical analysis*

Somatic tissues were analyzed for two mitochondrial parameters: total and mutant mtDNA. Germinal tissues were analyzed for the two aforementioned parameters as well as the expression of Ndufs4, Mt-nd2 and Mt-nd4 genes. There were three independent variables: Period (age), condition (in vitro, in vivo) and stage of development (GV M-II, 2-cell and blastocyst). The statistical analysis was designed to examine the effect of each independent variable on the study variables. All analysis were carried out using SAS v. 9.2 (Cary, N.C.) and the level of statistical significance was set at 0.05 throughout. Details are provided below for each model.

Data on total mtDNA copies and gene expression were log10 transformed to normalize the distribution. The mixed linear model, with animal identification (id) as a random factor (to take into account the multiple, non-independent measurements for each individual) and period, stage of development and condition as fixed factors. The mixed linear model is a generalization of the ANOVA that can include all types of independent variables. A mixed model includes independent variables for which we make predictions (fixed factors) and independent variables that are only needed to control for extraneous variation that is not of interest in itself (random factors). This was essential here because each individual provided multiple data points. We used a priori contrasts to compare pairs of means between levels of the independent variables. Given that there are multiple

contrasts, the alpha level for each contrast was adjusted with the Bonferroni correction to ensure a familywise error rate of 0.05.

The number of mutant mtDNA copies was analyzed, taking into account the total number of wild mtDNA copies; this was thus equivalent to modeling the percentage of deleted copies, thereby increasing power. A negative binomial regression model was used to compensate for the heavy right skew in the data (many zeros, embryonic samples without mtDNA common deletion). The negative binomial regression model is appropriate for counting data that deviate substantially from normality (ALLISON 1999). As in the above model, we considered both fixed and random factors. The fixed factors were: period, stage of development and condition. In the regression model, the number of copies was used as an offset as it varies from one individual to another, and animal identification (id) as a random factor to control for multiple measurements for each individual (ALLISON 1999). We also performed a priori contrasts to compare the expected number of deleted copies between levels of the independent variables. In the analysis of the effect of culture conditions in 2-cell embryos, the Wilcoxon two-sample test was utilized to compare the median of the groups. The Kruskal-Wallis non-parametric test was used in the analysis of the effects of developmental stage in the percentage of mtDNA common deletion, in order to compare the median of 2-cell and blastocyst (DAWSON and TRAPP 2001).

## 3.4. Results

### *3.4.1. Female fertility decreases with age*

Female fertility is known to decrease with aging but few studies have measured the effects of aging experimentally in mice (DUNCAN *et al.* 2009; HAMATANI *et al.* 2004; MANOSALVA and GONZÁLEZ 2009; SELESNIEMI *et al.* 2011). To examine the effect of aging in our C57BL/6 colony, females from three age groups, i.e. young (younger than 8 month), middle (between 8 and 16 months) and old (older than 16 months), were caged with C57BL/6 fertile males and checked for pregnancy during 3 months (Figure 1a). In the young group (n=25), 92% were fertile compared to 48% (n=29) in the middle and only 14% (n=7) in the old groups ($X^2 = 18.68$; $p < 0.00001$). These results clearly support the association of female aging with the gradual decrease in fertility in mice.

### *3.4.2. Tissue mtDNA integrity is compromised with age*

Previous studies have reported the presence of a mtDNA common deletion in several tissues (BRENNER *et al.* 1998; CORTOPASSI and ARNHEIM 1990; GIBSON *et al.* 2005; HSIEH *et al.* 2004; HSIEH *et al.* 2002; TANHAUSER and LAIPIS 1995), which in the case of somatic tissues seems to increase with aging (COOPER *et al.* 1992; CORRAL-DEBRINSKI *et al.* 1992a; CORRAL-DEBRINSKI *et al.* 1992b; CORTOPASSI and ARNHEIM 1990; CORTOPASSI

*et al.* 1992; LEE *et al.* 1994; LINNANE *et al.* 1990; SEIFER *et al.* 2002; SIMONETTI *et al.* 1992; SOONG *et al.* 1992). To further confirm these findings and to better evaluate the accumulation of common deletion in mice, we developed a quantitative real-time PCR assay to evaluate the ratio of deleted versus total mtDNA copies in different somatic tissues during aging (Figure 1b-c). Although the average copy numbers of mtDNA per genome was significantly higher ($p < 0.001$) in muscle (mean 2503 ± 262; range 995 to 4490) than in brain (mean 955 ± 117; range 225 to 2150) and liver (mean 950 ± 61; range 572 to 1324), no differences were observed within tissues among the three age groups (Figure 2). However, a significant increase in the percentage of mtDNA deletions was shown in tissues obtained from females at later periods in life. In all three tissues analyzed, the percentage of deleted copies was lower in young than middle ($p < 0.01$) and old age ($p < 0.01$) while there was no significant difference between middle and old age groups (Figure 2). Moreover, a significant positive correlation ($p < 0.05$) was observed between the ratio of deleted mtDNA and female age in brain ($R^2 = 0.32$), liver ($R^2 = 0.53$) and muscle ($R^2 = 0.33$). These results indicate that, while mtDNA copy numbers are not altered in old mice, mtDNA deletions accumulate continuously during aging, suggesting that tissue mtDNA integrity is compromised with age in both mitotically active (liver) and inactive (muscle and brain) tissues.

### *3.4.3. Cumulus and oocyte mtDNA are unaltered by aging*

Having identified increases in the proportion of deleted mtDNA in somatic tissues during aging, we decided to verify whether similar changes occurred in the somatic and germ cell compartments of the ovarian follicle. Therefore, we analyzed the effects of age on the mtDNA of GV-stage oocytes and their surrounding cumulus cells. Surprisingly, cumulus cell mtDNA showed no age-related increase in mtDNA deletion, which contrasts with our previous findings in other somatic tissues, i.e. brain, muscle and liver (Figure 3a-b). Moreover, compared to the somatic tissues analyzed previously, cumulus contained significantly fewer copies of mtDNA per genome and over $10^3$ times more mtDNA deletions (Figure 4). We also found no significant effects of aging on total and deleted copies of mtDNA in intra-follicular fully grown GV-stage oocytes (Figure 3c-d). Since we were unable to obtain ovulated oocytes from the oldest age group, we only analyzed young and middle-aged females. MII-stage oocytes from these two groups showed no significant changes in total mtDNA or in deleted mtDNA haplotypes (Figure 3e-f). To compare the effects of age in oocytes of all age groups, we cultured GV-stage oocytes and obtained MII-stage oocytes for analysis. As described in the in vivo group, no changes in the quantity of mtDNA haplotypes or in the percentage of deleted mtDNA haplotypes was observed with aging, which confirms that oocytes do not increase the percentage of deleted mtDNA haplotypes with aging (Figure 3g-h). Together, these results indicate that both cumulus cells and GV and MII oocytes are unaffected by aging as to mtDNA quantity and quality.

### *3.4.4. Mitochondrial-encoded transcripts are down-regulated in GV and up-regulated in MII-oocytes from aged females*

To further verify whether mitochondrial gene transcripts in oocytes were affected by aging, we analyzed the transcript levels of the nuclear-encoded mitochondrial gene, i.e. NADH dehydrogenase (ubiquinona) Fe-S protein 4 (Ndufs4), and the mitochondrial-encoded NADH dehydrogenase 2 (Mt-nd2) and Mt-nd4, all genes encoding proteins involved in the OXPHOS. In GV–stage oocytes, Ndufs4 and Mt-nd2 were unaffected by aging whereas the mitochondrial-encoded Mt-nd4 transcripts were significantly reduced in females with increased ages (Figure 5). In contrast, although Ndufs4 transcripts were unaffected by aging, ovulated MII-stage oocytes obtained from middle age females (older mice did not respond to the ovarian stimulatory regime) contained more mitochondrial gene transcripts than oocytes from the young group, indicating that mitochondrial gene transcription was up-regulated in MII oocytes. However, GV oocytes that underwent meiotic maturation in vitro show no age-related increase in transcript levels for any of the mitochondrial genes analyzed, indicating that the transcription was affected when GV oocytes attained meiotic resumption in vitro. Together, these results indicate that mitochondrial-encoded genes are down-regulated in GV-stage oocytes and up-regulated after in vivo meiotic resumption in aged females. However, in vitro maturation culture conditions seem to inhibit the up-regulation of mitochondrial gene transcripts in oocytes derived from middle age and old females.

### *3.4.5. In vitro culture of embryos during preimplantation development causes the up-regulation of mitochondrial gene transcripts*

Due to the transcriptional effects on oocyte in vitro maturation, we decided to further our investigation on the exposure of early embryos to in vitro culture systems. For such, we compared the abundance of mitochondrial gene transcripts in blastocysts stage embryos recovered either in vivo from the uterus of fertile young females or culturing oviductal zygotes in vitro to the blastocyst stage. Although no effects were observed on the copies of mtDNA or in the percentage of mtDNA deletions at any stage of development (data not shown), culture in vitro to the blastocyst stage increased the amount of mitochondrial gene transcripts (Figure 6), particularly of the mitochondrially-encoded gene Mt-nd2 ($p = 0.02$) and Mt-nd4 ($p = 0.008$). Therefore, in contrast to the inhibitory effect of in vitro culture during meiotic resumption (Figure 5), transcription of mitochondrial-encoded genes appears to be up-regulated by in vitro culture during the early stages of preimplantation development.

### *3.4.6. Mitochondrial deletions increase during meiosis and are then eliminated during early embryogenesis*

Compared to somatic cell mtDNA, where the percentage of the common deletion is generally below 1:100,000, oocytes seem to contain elevated ratios of deleted mtDNA haplotypes, which is unexpected due to their unique role as exclusive suppliers of mtDNA to the offspring. To examine the persistence of deleted mtDNA haplotypes during meiotic resumption and at post fertilization stages, we examined the presence of the mtDNA common deletion during meiosis and early embryo development. Samples were collected in vivo from young females at four stages of development: GV oocytes, M-II oocytes, 2-cell embryos and blastocyst embryos and analyzed the same five mitochondrial parameters as above. Total mtDNA copy number did not vary significantly between the ovulated M-II oocyte stage and the blastocyst stage (Figure 7), confirming previous reports from our and other laboratories that mtDNA replication does not occur until after the blastocyst stage in mice (PIKÓ and TAYLOR 1987; SMITH and ALCIVAR 1993; THUNDATHIL *et al.* 2005). Nonetheless, a significantly lower mtDNA copy number was found in GV-stage oocytes when compared to blastocysts, which may be due to the recovery of oocyte from ovarian follicles that had not yet completed growth. As for the proportion of mtDNA with the common deletion, an increase by over 8 fold was observed between the GV and M-II stages, indicating that meiotic resumption triggers a significant production of deleted mtDNA haplotypes that cannot be explained by changes in the total copies of mtDNA.

Moreover, GV oocytes that were unable to complete meiotic resumption in vitro, which had therefore arrested at the M-I stage after undergoing GV breakdown, showed an even larger percentage of deleted mtDNA haplotypes (mean 21.0 ± 1.6%, range 12 – 26), 50 fold higher than the deletions level found at the GV stage. However and most surprisingly, mtDNA haplotypes carrying the common deletion were completely absent in 2-cell and blastocyst stage embryos, indicating that deleted haplotypes present in the meiotic oocyte were eliminated early after fertilization and first cleavage. Together, these results indicate that meiotic resumption is accompanied by an increase in mtDNA deletions and those oocytes that are unable to complete meiotic resumption and polar body extrusion show even greater accumulation of defective mtDNA haplotypes. Nonetheless, deletions were no longer detected at the 2-cell stage and beyond, suggesting the existence of a mechanism to clear the cytoplasm of undesirable mtDNA haplotypes after fertilization. Although Ndufs4 transcripts, a mitochondrial nuclear-encoded gene, were significantly more abundant in GV than at later stages, transcripts of both mitochondrial-encoded genes, Mt-nd2 and Mt-nd4, became less abundant after meiotic resumption (M-II), increased significantly at the 2-cell stage to then become less abundant again at the blastocyst stage (Figure 7).

### 3.5. Discussion

It has been demonstrated that fertility declines with aging (LÉVY 2005; TATONE *et al.* 2006) and that oocyte developmental potential also declines with aging in vitro and in vivo (LÉVY 2005). However, until now, no direct relationship between mitochondrial mutations in oocytes and reproductive success has been demonstrated (MAY-PANLOUP *et al.* 2005).

Cassano and colleagues have reported the effects of aging on the copy number of mtDNA in various tissues in rats (liver and soleus muscle) (CASSANO *et al.* 2006). The mtDNA common deletion has been correlated with aging in a great variety of tissues by different researchers (CASSANO *et al.* 2004; HOFHAUS *et al.* 2003; HSIEH *et al.* 1994). Furthermore, higher deletion levels were found in non-dividing tissues than mitotic tissues (CORTOPASSI *et al.* 1992). The same deletion was also found at low percentages in mouse somatic tissues (less than 0.01%) (TANHAUSER and LAIPIS 1995).

In human granulosa cells, higher levels of mtDNA common deletion have been observed in women older than 38 years compared with younger women (SEIFER *et al.* 2002). Chan and colleagues, working with unfertilized human M-II oocytes found that women ≥ 35 years of age had higher incidence of mitochondrial common deletion and lower mtDNA copy number when they were compared with younger women (CHAN *et al.*

2005). In the analysis of human mtDNA deletions (included the mtDNA common deletion) relationship between these deletions and the age of oocytes and embryos were not found (BARRITT *et al.* 1999; BARRITT *et al.* 2000; BRENNER *et al.* 1998; CHEN *et al.* 1995; MÜLLER-HÖCKER *et al.* 1996).

In microarray experiments in mice, when young (5- to 6-week-old) and old (42- to 45-week-old) M-II oocytes were compared, genes such as Mt-nd3, ATPase6 and COI-COIII, encoded in the mitochondrial genome and involved in mitochondrial electron transport chain, were more highly expressed in old oocytes. In contrast, genes encoded in the nuclear genome and related to 'energy pathways' and mitochondrial function were more highly expressed in young oocytes (HAMATANI *et al.* 2004). In human unfertilized oocytes and arrested embryos, expression of genes inside the common deletion was lower than genes outside this deletion (HSIEH *et al.* 2004). Actually, it was reported that gene expression patterns of OXPHOS complex I subunits are organized in clusters and all mtDNA-encoded complex I share a similar expression pattern (GARBIAN *et al.* 2010).

Kameyama and colleagues compared the mtDNA copy numbers between rat embryos in vivo and in vitro. They did not observe significant differences in 2-cell and 8-cell embryos but the number of mtDNA copies was lower in morula and blastocyst stages in vivo compared with in vitro embryos (KAMEYAMA *et al.* 2007). Zeng and colleagues

found lower quantities of mtDNA copy number in oocytes maturated in vitro than in vivo from rat ovarian follicles of different sizes (ZENG *et al.* 2009).

In early experiments, Pikó and Taylor found in mouse embryos (from one-cell to the blastocyst stage) constant levels of mtDNA, around 119,000 copies per embryo (PIKÓ and TAYLOR 1987). More recently, McConnell and Petrie reported that there is a short period of mtDNA synthesis immediately after fertilization (MCCONNELL and PETRIE 2004). A significant reduction of mtDNA rearrangements from oocytes to embryos has been reported by many researchers (BARRITT *et al.* 1999; BRENNER *et al.* 1998; HSIEH *et al.* 2002).

Autophagy is a highly organized and specific intralysosomal protein degradation pathway. It takes several forms: macroautophagy (autophagy of large organelles, i.e. mitochondria), microautophagy (autophagy of small organelles) and the chaperone-mediated autophagy (CMA, selective digestion of proteins) (TERMAN *et al.* 2007). The autophagy plays a critical role during preimplantation embryonic development. The level of autophagy is low in unfertilized oocytes but is activated four hours after fertilization (TSUKAMOTO *et al.* 2008a). The ability to select against deleterious mutations in mtDNA has been reported in mice. Somatic cells package multiple mtDNA genomes in a single nucleoid complex. These cells have a more complex organization of the nucleoids than oocytes. This fundamental difference suggests that oocytes may be better able to detect and

remove defective mtDNA genomes than somatic cells, possibly due in part to the simpler organization of the mtDNA in smaller nucleoids (BOGENHAGEN 2010).

Data of many articles suggest that aerobic respiration is the main source of energy during oocyte maturation and embryo implantation, but anaerobic respiration predominates during early preimplantation embryo development (WILDING *et al.* 2005). More recently, it was proposed that the levels of ATP required for embryo development, implantation and birth are supplied by both, aerobic and anaerobic respiration, in a synchrony fashion. Aerobic respiration is present during human embryo development and the excess of energy requirements are supplied through anaerobic pathway (WILDING *et al.* 2009).

In our experiments, we observed that female fertility decreased with aging. The aging did not affect the copy number of mtDNA, but increased the percentage of mitochondrial deletions in somatic tissues such as brain, liver and muscle. By contrast, the cumulus cell and oocyte mtDNA were unaltered by aging. Within somatic cells, the cumulus cells presented the lowest quantity of mtDNA copy number and the highest percentage of deleted mtDNA. The mitochondrial-encoded transcripts were down-regulated in GV oocytes and up-regulated in M-II oocytes from aged females. In vitro culture during preimplantation embryonic development caused up-regulation of mitochondrial gene transcripts. MtDNA deletions increased during oocyte maturation, and were later eliminated during early embryogenesis.

We did not observe significant effects of aging in the total copy numbers of mtDNA in mouse tissues including brain, liver and muscle. In our analysis of three somatic tissues: brain, liver and muscle; we found the lowest ratio of deletion in young brains (0.000075%) and the highest in middle-age muscle cells (0.00192%). These deletion levels were similar to those reported by Tanhauser and Laipis (1995). As to the percentage of mtDNA common deletion, we observed that this deletion increased with aging in somatic tissues: brain, liver and muscle; similarly to that reported by Cassano *et al*. (2004). However, we did not observe, in disagreement with Cortopassi *et al* (1992), a difference between non-dividing and mitotic tissues.

In our experiments, we did not observe significant effects of aging on the percentage of mtDNA common deletion in mouse granulosa cells. The granulosa cells were different from other somatic cells; they had the lowest copy numbers of mtDNA and the highest percentage of mtDNA common deletion. In our experiments, we did not find significant effects of aging in total mtDNA copies and percentage of mitochondrial common deletion in fully grown GV and M-II oocytes. These results were consistent with previous reports (BARRITT *et al.* 1999; BARRITT *et al.* 2000; BRENNER *et al.* 1998; CHEN *et al.* 1995; MÜLLER-HÖCKER *et al.* 1996). It is worth noting that we were not able to obtain old M-II oocytes (in vivo) from oviducts but, we were able to obtain old M-II oocytes (in vitro) by in vitro maturation of GV oocytes. It will be interesting to test if these old M-II oocytes in vitro could be fertilized by in vitro fertilization and produce healthy offspring.

In our experiments, when the transcript abundance in M-II oocytes in vivo were compared, the age did not affect the expression of Ndufs4 gene (nuclear gene) between the young (4- to 32-week-old) and middle (32- to 64-week-old) females but, the age affected the expression of Mt-nd2 and Mt-nd4 (mitochondrial genes) with higher expression (both genes) in middle than young females. These last results were similar to those reported by Hamatani and colleagues (2004). We expected to have differences in the gene expression between Mt-nd2 (gene outside common deletion) and Mt-nd4 (gene inside common deletion) like reported by Hsieh *et al*. (2004). However, we did not find significant differences. Both genes presented similar expression patterns, probably because these two genes belong to the complex I and are organized in clusters, similarly as described in Garbian *et al*. (2010).

Our results were different from those reported by Kameyama *et al*. (2007) and Zeng *et al*. (2009). We did not find significant effects of culture in vitro on total mtDNA copy numbers, in M-II oocytes, 2-cell embryos and blastocyst embryos. The two mitochondrial genes (Mt-nd2, Mt-nd4) were up-regulated when the blastocysts were cultured in vitro. The nuclear-mitochondrial gene (Ndufs4) tended to increase in the same way but we did not find significant differences. Probably, in vitro, the embryos produce more energy by the aerobic mitochondrial pathway, and the cells increase the synthesis of proteins related with the complex I of OXPHOS.

In our experiments, we observed no significant differences in total mtDNA copy numbers among, M-II oocytes, 2-cell embryos and blastocyst embryos, similarly to what is reported by Pikó and Taylor (1987). However, our mean of mtDNA copies was higher (~ 260,648) than that reported by Pikó and Taylor and very close to that reported by Thundathil and colleagues (~ 256,000) (PIKÓ and TAYLOR 1987; THUNDATHIL *et al.* 2005). We did not observe an increase in mtDNA copy numbers after fertilization unlike reported by McConnell and Petrie (2004).

In our results, we observed a larger percentage of mtDNA common deletion in oocytes compared with embryos, consistent with previous reports (BRENNER *et al.* 1998; HSIEH *et al.* 2002). We did not observe a significant reduction of mtDNA rearrangements from GV oocytes to M-II oocytes as reported by Barritt and colleagues (1999); as a matter of fact, we observed an increase in the percentage of the mtDNA common deletion. Our results of higher levels of deletion in the oocyte stage of development could be explained by increased utilization of oxidative phosphorylation (aerobic respiration) in this phase, and consequently more production of ROS that can cause more mtDNA deletions. The reduction of deletion in 2-cell embryos could be explained by a decrease in aerobic respiration (mitochondrial) and an increase in anaerobic respiration (cytosolic) in this stage of development.

In our experiments, we observed an increase in the percentage of mtDNA common deletion from GV oocyte (0.418 %) to M-II oocytes (3.383 %) and after fertilization; no

deleted copies were seen in 2-cell and blastocyst embryos (0 %). This amazing finding may indicate that mammalian cells have another mitochondrial bottleneck system, for eliminating all the inconvenient mitochondria harboring a defective mtDNA, and this purifying method exists between the stages of M-II oocytes and 2-cell embryos, probably after fertilization. We suggested a mechanism of autophagy (TSUKAMOTO *et al.* 2008a; TSUKAMOTO *et al.* 2008b; WALLACE 2005) for the destruction of damaged mitochondria (harboring deleted mtDNA) or the elimination of the whole oocyte by apoptosis when the heteroplasmy levels exceed a maximum threshold of deletion (FAN *et al.* 2008; TAYLOR and TURNBULL 2005; WALLACE 2005).

In summary, in our experiments the mouse female fertility decreased with aging, but this decrease in fertility was not related to changes in the mtDNA from oocytes. The oocytes were not affected by the process of aging like somatic tissues were affected. The percentage of mtDNA common deletion increased with aging in somatic tissues. Granulosa cells are special somatic cells that were not affected by aging; within somatic cells, they presented the lowest number of total mtDNA copy numbers and the highest percentage of deleted mtDNA. The in vitro culture did not affect the quality of oocytes and embryos in most mitochondrial parameters analyzed. The preovulatory surge of luteinizing hormone (LH) results in increased mitochondrial metabolism (MAGNUSSON *et al.* 1977; MAGNUSSON *et al.* 1981), necessary for supplying the increased energy requirements in meiotic resumption. This increase in OXPHOS during oocyte maturation (VAN BLERKOM 2000; VAN BLERKOM *et al.* 1997) produce higher levels of ROS that can cause more mtDNA

common deletion. At the beginning of the embryonic development, OXPHOS is reduced and glycolysis is increased (BAVISTER and SQUIRRELL 2000; FACUCHO-OLIVEIRA and ST JOHN 2009; WILDING *et al.* 2009), this last anaerobic pathway occurs in the cytosol and does not involve the mitochondria. Probably, in M-II oocytes the mitochondria with higher percentages of deleted mtDNA are detected as damaged mitochondria, mainly because they have only one copy by mitochondrion. These damaged mitochondria are probably destroyed by a process of autophagy at the beginning of the embryonic period (TSUKAMOTO *et al.* 2008a; TSUKAMOTO *et al.* 2008b; WALLACE 2005) or the elimination of the whole oocyte by apoptosis (FAN *et al.* 2008; TAYLOR and TURNBULL 2005; WALLACE 2005). Because we did not find effects of aging in most mitochondrial parameters analyzed in oocytes, we suggest that mtDNA common deletion is more related to the cellular status of germinal cells (stage of development, energy requirements) than the process of aging.

Further experiments must be performed to better understand the roles of mitochondria in aging, meiosis, fertilization and early embryo development of germinal tissues, mainly to elucidate the ways in which the deleted mtDNA copies are eliminated at early embryonic stages.

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**Table 1. Primers utilized in quantification of total mtDNA, deleted mtDNA and gene expression of mouse oocytes and embryos**

| Name of primers | Purpose | Gene | Length (bp) | Tm | Sequence of primers (5' to 3') | Amplicon (bp) |
|---|---|---|---|---|---|---|
| musTOTUPL-F | Quantification of total mtDNA | Total mtDNA | 24 | 59 | CGA TTA AAG TCC TAC GTG ATC TGA | 77 |
| musTOTUPL-R | Quantification of total mtDNA | Total mtDNA | 27 | 59 | CTG GGA GAA ATC GTA AAT AGA TAG AAA | |
| ProbeTOT 85 (TAQMAN UPL) | Probe of total mtDNA | Total mtDNA | 8 | | TCC AGG TC | |
| musDELUPL-F | Quantification of deleted mtDNA | Deleted mtDNA | 22 | 60 | TGA CCA TTA ACT GGA GCC TTT T | 62 |
| musDELUPL-R | Quantification of deleted mtDNA | Deleted mtDNA | 22 | 59 | TGA GGT TGA TGA TGT TGG AGT T | |
| ProbeDEL 13 (TAQMAN UPL) | Probe of deleted mtDNA | Deleted mtDNA | 8 | | CCT CCT TC | |
| musNS2035-F1 | Standard curve of total mtDNA | Total mtDNA | 23 | 55 | ATA ATC ACT TGT TCC TTA ATT AG | 1337 |
| musNS3372-R1 | Standard curve of total mtDNA | Total mtDNA | 21 | 55 | CTA ATT CTG ATT CTC CTT CTG | |
| musNDUFS4-F | Relative quantification of gene expression | Ndufs4 | 22 | 56 | AGT TGA TGA GAA ACT GGA TAT C | 93 |
| musNDUFS4-R | Relative quantification of gene expression | Ndufs4 | 22 | 56 | ATT GCG AGC AGG AAC AAA GAT T | |
| NZB-F5 | Relative quantification of gene expression | Mt-nd2 | 24 | 66 | CGC CCC ATT CCA CTT CTG ATT ACC | 189 |
| NZB-R5 | Relative quantification of gene expression | Mt-nd2 | 24 | 65 | TTA AGT CCT CCT CAT GCC CCT ATG | |
| musND4-SP-F1 | Relative quantification of gene expression | Mt-nd4 | 21 | 76 | TAA TCT AGC TCT ACC CCC TTC | 256 |
| musND4-SP-R1 | Relative quantification of gene expression | Mt-nd4 | 22 | 68 | ACT GGT AGT TAG AAG AAT AAG T | |
| musGapd-F | Relative quantification of gene expression | Gapdh | 20 | 60 | TCC CAC TCT TCC ACC TTC GA | 100 |
| musGapd-R | Relative quantification of gene expression | Gapdh | 20 | 58 | TGT TGC TGT AGC CGT ATT CA | |
| Rab-alpha-globine-F | External control of gene expression | Rabbit α-1-globine | 20 | 67 | GCA GCC ACG GTG GCG AGT AT | 257 |
| Rab-alpha-globine-R | External control of gene expression | Rabbit α-1-globine | 20 | 60 | GTG GGA CAG GAG CTT GAA AT | |

**Table 2. Primers utilized in quantification of total and deleted mtDNA from mouse somatic tissues**

| Name of primers | Purpose | Gene | Length (bp) | Tm | Sequence of primers (5' to 3') | Amplicon (bp) |
|---|---|---|---|---|---|---|
| GPDH-F | Standard curve of Gapdh | Gapdh | 19 | 60 | TTG GCA GCA CCA GTG GAT G | 516 |
| GPDH-R | Standard curve of Gapdh | Gapdh | 20 | 60 | CCT TGA TAT GGT GCA ACC TG | |
| UPLmusGPDH-F | Quantification of number of genomes | Gapdh | 18 | 60 | CAA TGA GTG AGG TCC TGC | 73 |
| UPLmusGPDH-R | Quantification of number of genomes | Gapdh | 20 | 60 | TGG AAG GGC TCA TGG TAT GT | |
| Probe Gapdh (TAQMAN UPL) | Probe Gapdh | Gapdh | 8 | | CTG TCT CC | |
| musNS2035-F1 | Standard curve of total mtDNA | Total mtDNA | 23 | 55 | ATA ATC ACT TGT TCC TTA ATT AG | 1337 |
| musNS3372-R1 | Standard curve of total mtDNA | Total mtDNA | 21 | 55 | CTA ATT CTG ATT CTC CTT CTG | |
| musTOTUPL-F | Quantification of total mtDNA | Total mtDNA | 24 | 60 | CGA TTA AAG TCC TAC GTG ATC TGA | 77 |
| musTOTUPL-R | Quantification of total mtDNA | Total mtDNA | 27 | 60 | CTG GGA GAA ATC GTA AAT AGA TAG AAA | |
| ProbeTOT 85 (TAQMAN UPL) | Probe total mtDNA | Total mtDNA | 8 | | TCC AGG TC | |
| T7 | Standard curve of deleted mtDNA | Deleted mtDNA | 20 | 50 | ATT ATG CTG AGT GAT ATC CC | 240 |
| SP6 | Standard curve of deleted mtDNA | Deleted mtDNA | 21 | 50 | TAA ATC CAC TGT GAT ATC TTA | |
| musDELUPL-F | Quantification of deleted mtDNA | Deleted mtDNA | 22 | 60 | TGA CCA TTA ACT GGA GCC TTT T | 62 |
| musDELUPL-R | Quantification of deleted mtDNA | Deleted mtDNA | 22 | 60 | TGA GGT TGA TGA TGT TGG AGT T | |
| ProbeDEL 13 (TAQMAN UPL) | Probe deleted mtDNA | Deleted mtDNA | 8 | | CCT CCT TC | |

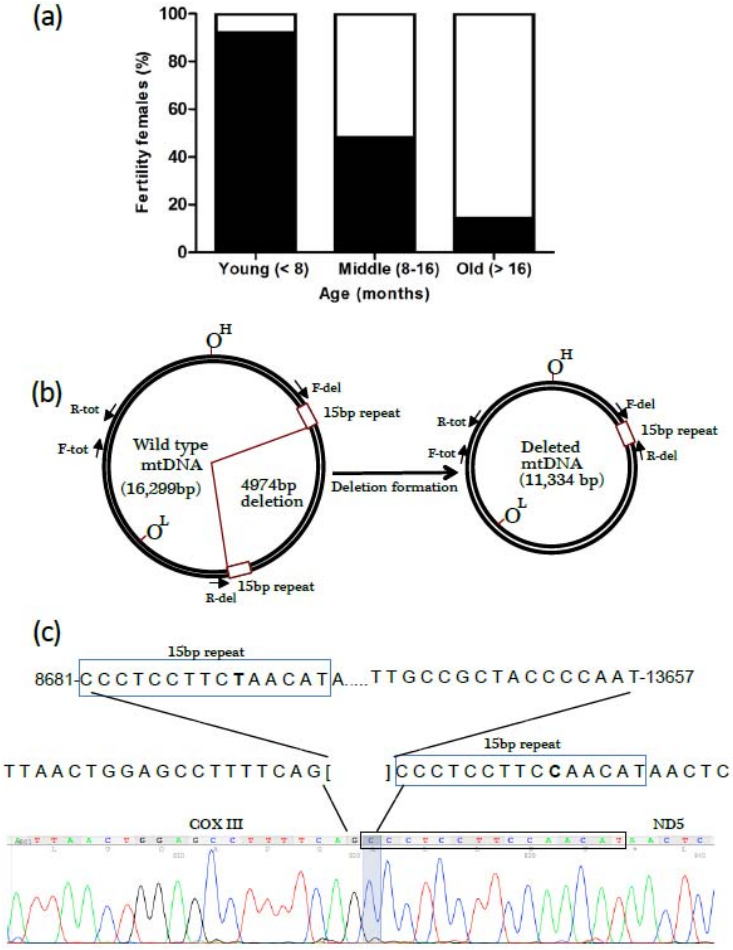


**Figure 1. Loss of female fertility with age in mice and detection of the mtDNA 4974bp deletion in mice**. (a) Percentages of females from three age groups (young, middle and old) that established (fertile; black) or not (infertile; white) full-term gestations after caging with fertile males for three months. (b) Mouse mitochondrial genome with and without the 4974bp deletion; the position of PCR primers for detecting this deletion were F-del and R-del. The fragment was too large for wild-type mtDNA (>5 kb) to be amplified with the PCR conditions used for detection. Total mtDNA copy number was assessed using PCR primers F-tot and R-tot. (c) Confirmation of the 4974 bp deletion. Representative sequence profile of the PCR products from the tissues carrying the deletion. Based on the article of Chen and colleagues (CHEN *et al.* 2011).

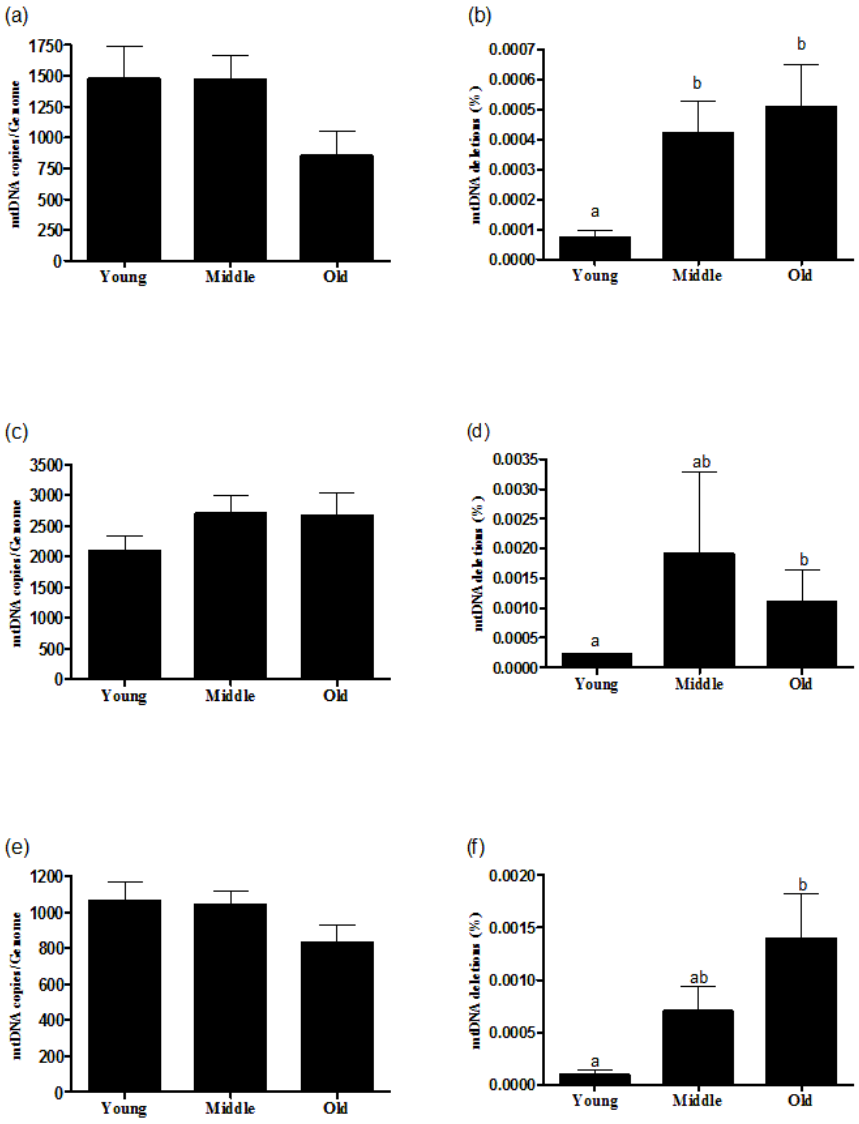


**Figure 2**. **Quantification of normal and deleted mtDNA haplotypes in somatic tissues**. Total mtDNA copy numbers and the respective mtDNA common deletion percentage in brain (a-b), muscle (c-d) and liver (e-f) in female mice of different ages. Black boxes represent mean values and vertical bars the standard errors of the means. Different subscripts represent significant differences ($p \leq 0.05$).

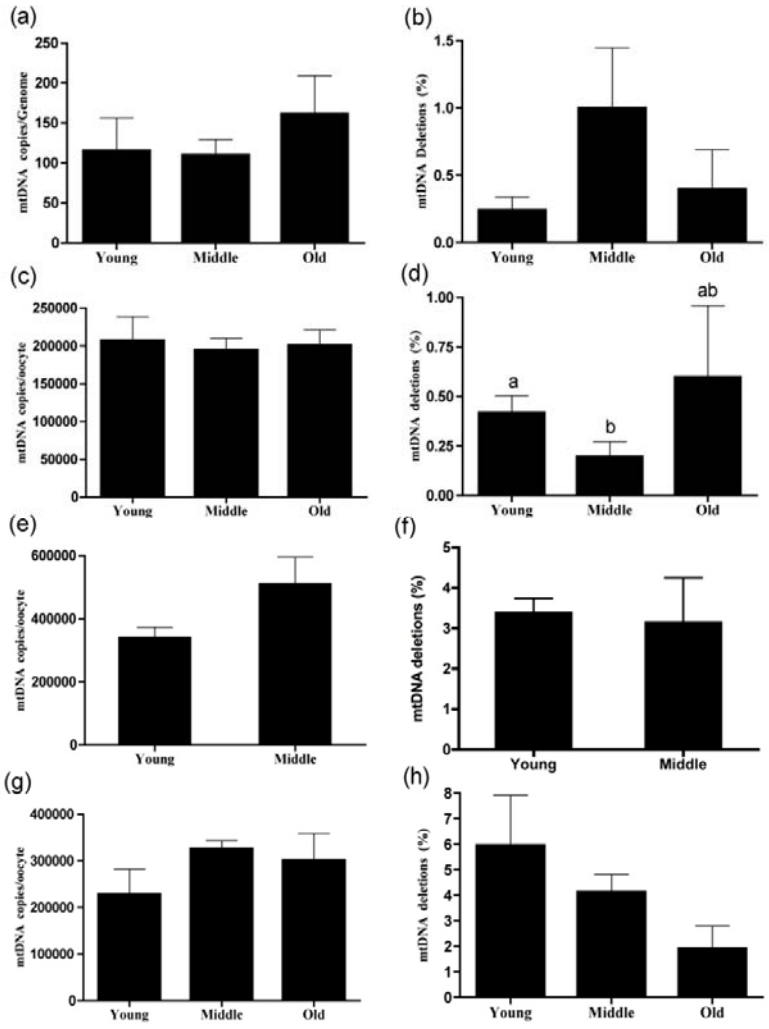


**Figure 3**. **Quantification of normal and deleted mtDNA haplotypes in cumulus and oocytes produced in vivo and in vitro.** Total mtDNA copy numbers and the respective mtDNA common deletion percentage in cumulus cells (a-b), germinal vesicle-stage (c-d), in vivo-derived (e-f), and in vitro-derived metaphase II-stage (g-h) oocytes from female mice of different ages. Black boxes represent mean values and vertical bars the standard errors of the means. Different subscripts represent significant differences ($p \leq 0.05$).

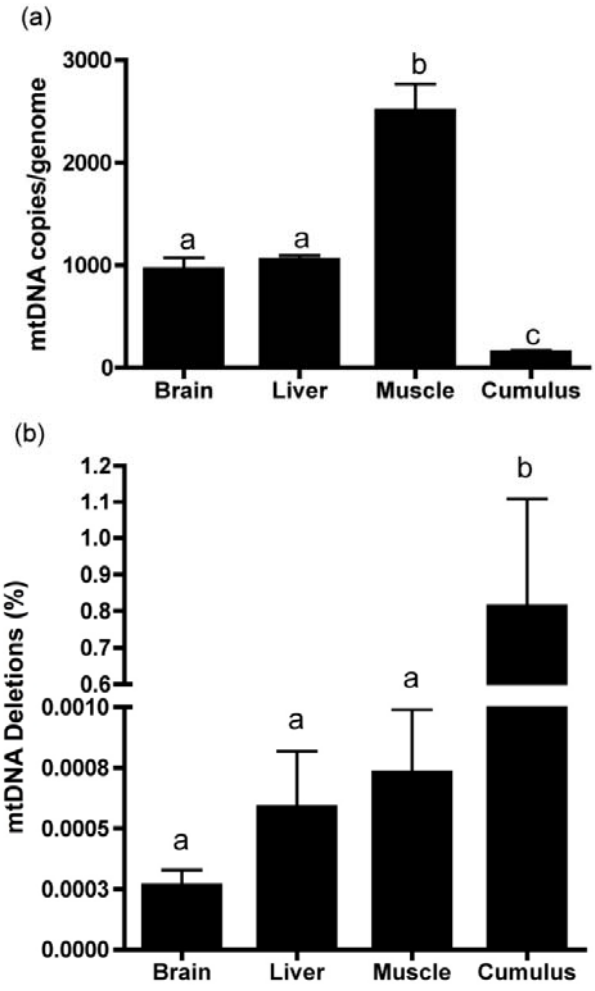


**Figure 4**. **Quantification of normal and deleted mtDNA haplotypes in different somatic tissues.** Comparison of (a) total mtDNA copy numbers and (b) mtDNA common deletion percentage between brain, liver, muscle and cumulus cells. Black boxes represent mean values and vertical bars the standard errors of the means. Different subscripts represent significant differences ($p \leq 0.05$).

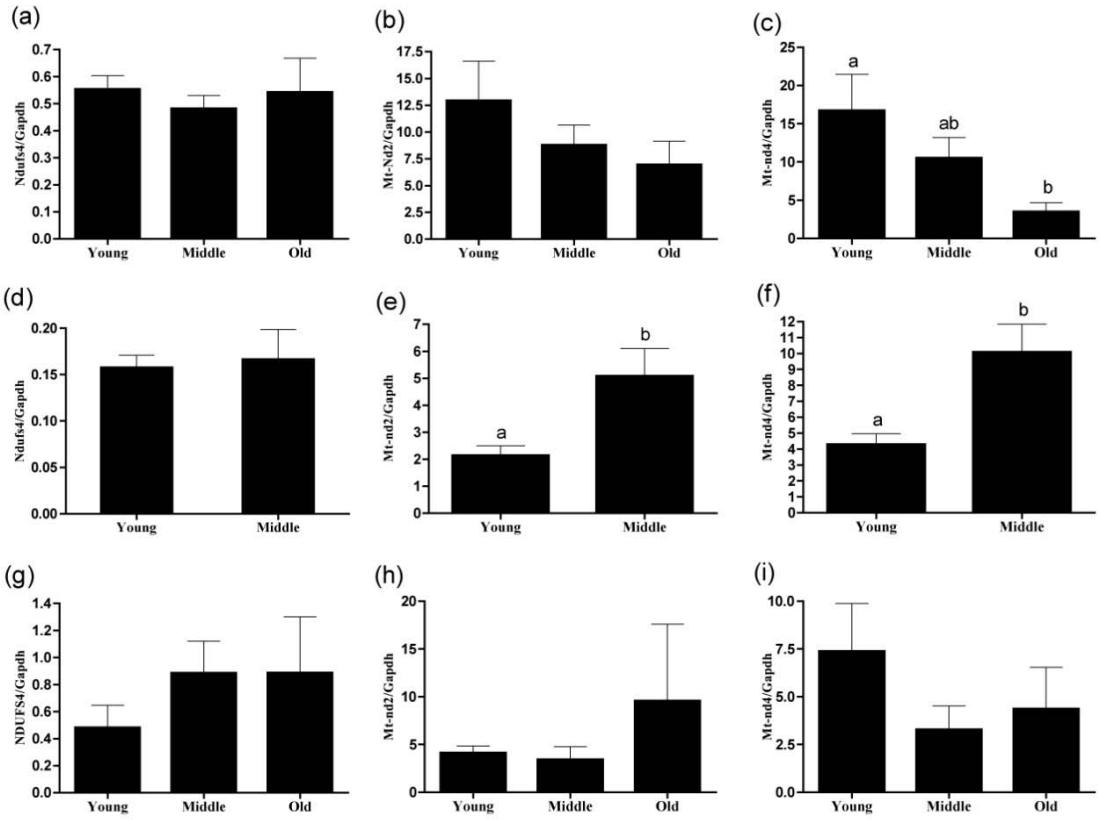


**Figure 5**. **Mitochondrial gene transcript abundance in oocytes from aged females.** Relative quantification of mitochondrial gene transcripts in germinal vesicle (a-c), in vivo- (d-f) and in vitro-derived metaphase-stage (g-i) oocytes. Transcript abundance measurements (relative to the housekeeping gene Gapdh) were obtained for the nuclear-encoded mitochondrial gene Ndufs4 (a,d,g), and the mitochondrially-encoded Mt-nd2 (b,e,h) and Mt-nd4 (c,f,i) genes. Black boxes represent mean values and vertical bars the standard errors of the means. Different subscripts represent significant differences ($p \leq 0.05$).

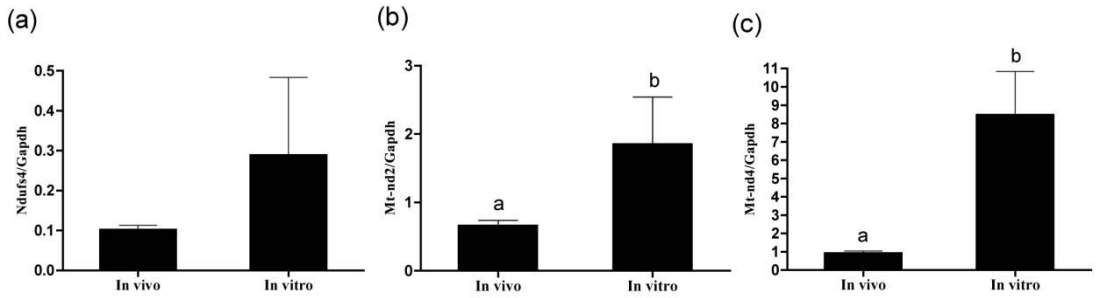


**Figure 6**. **Mitochondrial gene transcript abundance in blastocysts derived in vitro.** Relative quantification of mitochondrial gene transcripts in blastocyst stage embryos derived either directly from the reproductive tract (in vivo) or after culture in vitro for 3.5 days from the zygote stage. Transcript abundance measurements (relative to the housekeeping gene Gapdh) were obtained for the nuclear-encoded mitochondrial gene Ndufs4 (a), and the mitochondrially-encoded Mt-nd2 (b) and Mt-nd4 (c) genes. Black boxes represent mean values and vertical bars the standard errors of the means. Different subscripts represent significant differences ($p \leq 0.05$).

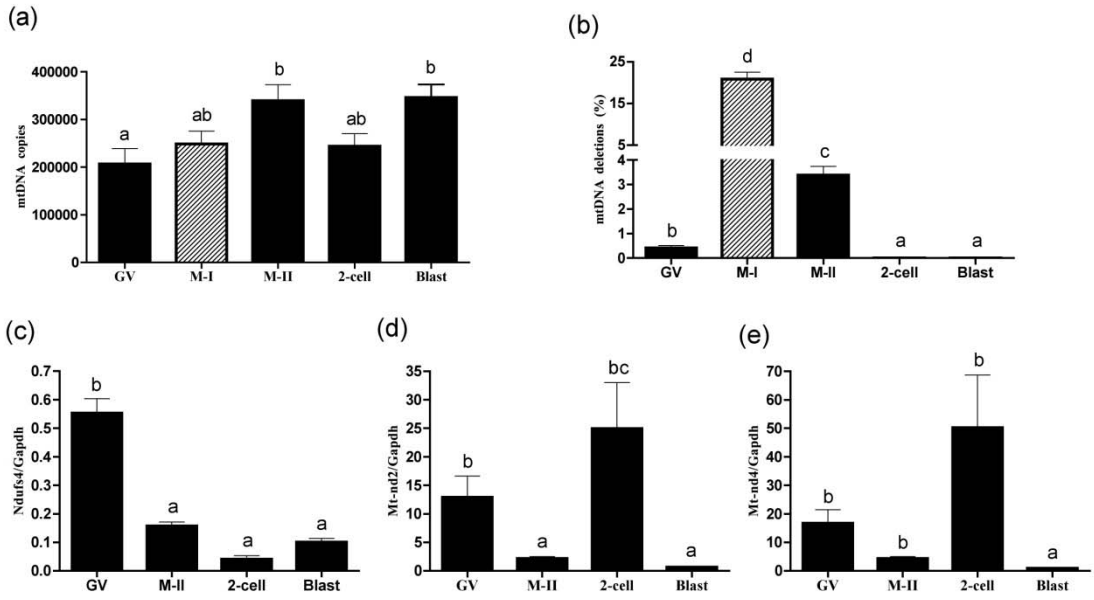


**Figure 7**. **MtDNA and transcript alterations in oocytes and early embryos.** (a) Total mtDNA copy number, (b) percent mtDNA deletions, (c) and relative quantification of mitochondrial gene transcripts in *in vivo*-derived germinal vesicle (GV), metaphase-stage of first (M-I; grey box) and second (M-II) meiosis, 2-cell and blastocyst (Blast) stage embryos. Transcript abundance measurements (relative to the housekeeping gene Gapdh) are presented for the nuclear-encoded mitochondrial gene Ndufs4 (a,d,g), and the mitochondrially-encoded Mt-nd2 (b,e,h) and Mt-nd4 (c,f,i) genes. Boxes represent mean values and vertical bars the standard errors of the means. Different subscripts represent significant differences ($p \leq 0.05$).

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# General discussion

The mtDNA showed different patterns of segregation in our SCNT and aging experiments. The artificial heteroplasmy produced at moment of oocyte reconstruction in bovine somatic cell nuclear transfer was more stable than the natural heteroplasmy produced by aging in germinal and somatic tissues. In our experiments, the artificial heteroplasmy was produced by an insertion (66 bp) in the D-loop of the bovine mitochondrial DNA, and the natural heteroplasmy was produced by a huge deletion (4974 bp) in the coding region of the mouse mitochondrial DNA. It has been described in other experiments; that insertions are the main mtDNA mutations transmitted by heredity. Spontaneous mtDNA mutations are primarily deletions (WALLACE 2005).

The introduction of foreign mitochondria from donor cell at the moment of oocyte reconstruction in somatic cell nuclear transfer has produced different heteroplasmy outcomes in cloned progenies. In some nuclear transfer (NT) experiments, only homoplasmy from the mtDNA of the recipient oocyte were found (DO *et al.* 2001; EVANS *et al.* 1999; HIENDLEDER *et al.* 2004; TAKEDA *et al.* 1999), in other NT experiments only heteroplasmic clones were found at different proportions (HAN *et al.* 2004; HIENDLEDER *et al.* 1999; LLOYD *et al.* 2006; MA *et al.* 2008; ST. JOHN and SCHATTEN 2004; STEINBORN *et al.* 1998; TAKEDA *et al.* 2008; TAKEDA *et al.* 2006), and in other NT experiments both results were found in the same experiments: homoplasmy from the recipient cell and heteroplasmy from the two cells in different percentages (BURGSTALLER *et al.* 2007; DO *et al.* 2002; FERREIRA *et al.* 2007; HIENDLEDER *et al.* 2003; MEIRELLES *et al.* 2001;

SANSINEMA *et al.* 2011; STEINBORN *et al.* 2002; STEINBORN *et al.* 2000; TAKEDA *et al.* 2003). Only in a few experiments have reported homoplasmic results from donor cell mtDNA (CHEN *et al.* 2002; SMITH *et al.* 2000).

In the results of our experiments with bovine SCNT, heteroplasmy levels were maintained close to the neutral segregation (˜ 0.9%, Steinborn *et al*. 2002) in all the stages of development, all the periods of life and in all the tissues, regardless of the origin (placenta or fetus) or the cell division status (mitotic or post-mitotic) of the tissues. Only five tissues analyzed from one clone presented higher levels of heteroplasmy (~ 20.85%) and were excluded of the general statistical analysis because the clone was considered an outlier.

In SCNT experiments, when recipient oocytes are obtained from slaughterhouse ovaries or from superovulated unrelated cows, cloned progeny do not share the same mitochondria and may differ by cytoplasmic genetic effects (HIENDLEDER *et al.* 1999). These differences in the cytoplasmic sources can increase the chance to produce divergent donor mtDNA segregation patterns in somatic cell nuclear transfer experiments (BURGSTALLER *et al.* 2007).

In our SCNT experiments, even though we worked with slaughterhouse oocytes and could not know if donor and recipient cells shared the same mitochondrial DNA, we observed a consistent neutral segregation of mtDNA in most analyzed tissues. This

phenomenon could be explained by the fusion of the mitochondria from oocyte and the somatic cell (fibroblast) at the moment of the electrofusion. Probably, after the electrofusion, the two types of mtDNAs share the same nucleoid population and the nucleoids were distributed in an equitable fashion during all the mitotic divisions since zygote and until adulthood life, producing the mitochondrial neutral segregation observed in most produced SCNT clones. If the mitochondria did not fuse at moment of electrofusion, the mitochondrial DNA stayed in different nucleoid populations and the mitochondrial segregation was stochastic producing results of higher levels of heteroplasmy like was observed in the outlier clone in our SCNT experiments. The reasons of the neutral and stochastic segregation of the mtDNA were described by Jacobs and colleagues (JACOBS *et al.* 2000).

In our second series of experiments, we analyzed the heteroplasmy in mouse females produced by a natural deletion in the mitochondrial DNA called the "common deletion". We measured this deletion in germinal and somatic tissues to elucidate the effects of aging, stage of development and culture in vitro. This mitochondrial mutation is a huge deletion of 4977 bp length in humans (HOFHAUS *et al.* 2003) and 4974 bp in mouse (TANHAUSER and LAIPIS 1995) deleting 7 genes (Mt-nd5, Mt-nd4, Mt-nd4L, Mt-nd3, COIII, ATPase6 and ATPase8) of 13 mitochondrial genes encoding proteins of the OXPHOS (HSIEH *et al.* 2004).

This mtDNA common deletion has been found in somatic and germinal tissues. This deletion has been correlated with aging in somatic tissues, but not in germinal tissues (BRENNER *et al.* 1998; COOPER *et al.* 1992; CORRAL-DEBRINSKI *et al.* 1992a; CORRAL-DEBRINSKI *et al.* 1992b; CORTOPASSI and ARNHEIM 1990; CORTOPASSI *et al.* 1992; GIBSON *et al.* 2005; HSIEH *et al.* 2004; HSIEH *et al.* 2002; LEE *et al.* 1994; LINNANE *et al.* 1990; SEIFER *et al.* 2002; SIMONETTI *et al.* 1992; SOONG *et al.* 1992; TANHAUSER and LAIPIS 1995).

In our experiments of heteroplasmy in female mice, the effects of aging were different for somatic and germinal tissues. In somatic tissues (brain, liver and muscle), the total mtDNA copies did not increase with aging but the heteroplasmy measured as mtDNA "common deletion" increased with aging. Aging did not affect the heteroplasmy in granulosa cells. In germinal tissues, aging did not affect total and deleted mitochondrial DNA copy numbers in GV and M-II oocytes. In M-II oocytes in vivo, aging did not affect the expression of the nuclear-mitochondrial gene Ndufs4 but affected the two mitochondrial genes Mt-nd2 and Mt-nd4 (more expression in middle than young period), in agreement with Hamatani and colleagues (HAMATANI *et al.* 2004). Contrary to what we expected, the expression levels of Mt-nd2 (gene outside common deletion) was similar to that of Mt-nd4 (gene inside common deletion), probably because these two genes belong to complex I of oxidative phosphorylation and they are organized in clusters, as described by Garbian and colleagues (GARBIAN *et al.* 2010).

We did not observe differences in total mtDNA copy numbers among oocytes and embryos. We did not observe the short period of mtDNA synthesis after fertilization reported by McConnell and Petrie (MCCONNELL and PETRIE 2004). In our results, upon the resumption of meiosis, the percentage of mtDNA common deletion increased from GV to M-II oocyte, with huge percentage (3.383 %) of deleted mtDNA in this last stage of oocyte. After fertilization, this enormous level of heteroplasmy disappeared in two-cell and blastocyst stages. Probably, there is a protective mechanism to avoid the presence of deleted copies at beginning of the embryonic life. We suggest a mechanism of autophagy (TSUKAMOTO *et al.* 2008a; TSUKAMOTO *et al.* 2008b; WALLACE 2005) for the destruction of damaged mitochondria (harbouring deleted mtDNA) or the elimination of the whole oocyte by apoptosis when the heteroplasmy levels exceed a maximum threshold of deletion (FAN *et al.* 2008; TAYLOR and TURNBULL 2005; WALLACE 2005).

The aerobic production of energy increases in the resumption of meiosis (MAGNUSSON *et al.* 1977; MAGNUSSON *et al.* 1981), this increase in OXPHOS during oocyte maturation (VAN BLERKOM 2000; VAN BLERKOM *et al.* 1997) produces higher levels of ROS, which can damage the mtDNA and produce more mitochondrial deletions (BARTMANN *et al.* 2004; HOFHAUS *et al.* 2003). After fertilization, the production of energy switches to the anaerobic pathway (glycolysis) (BAVISTER and SQUIRRELL 2000; FACUCHO-OLIVEIRA and ST JOHN 2009; WILDING *et al.* 2009) without production of ROS. In our experiments, the presence of mitochondrial DNA common deletion was increased in the resumption of meiosis and disappeared at beginning of the embryo development. Probably

in germinal tissues, this mitochondrial deletion is more related with the active pathway of energy production utilized in the cells than the process of aging.

In our experiments, the in vitro culture did not affect the total mtDNA copy numbers and the percentage of mtDNA common deletion in young M-II oocytes, 2-cell and blastocyst embryos. These results were different from those reported by Kameyama and colleagues (KAMEYAMA *et al.* 2007). The in vitro culture did not affect the expression levels of the nuclear-mitochondrial gene Ndufs4, but increased the expression levels of two mitochondrial genes Mt-nd2 and Mt-nd4 (higher in *in vitro* than *in vivo* conditions).

Two different sources of mutations in the mtDNA generated in normal or reconstructed oocytes produced different heteroplasmy outcomes at the beginning of the embryonic life. The first mutation, an insertion of 66 bp in the D-loop region of the mtDNA from a fetal fibroblast, introduced by artificially manipulation into an enucleated oocyte at the moment of reconstruction by the technique of bovine somatic cell nuclear transfer, was not eliminated and maintained in the same proportion from embryo to adult life. The second mutation, a huge mitochondrial deletion (4974 bp in mouse) affecting 7 genes of the coding region, showed an increase during the resumption of meiosis and subsequently disappeared at the beginning of the embryonic life, and maintained in the same levels until the blastocyst stage.

Probably, the somatic small insertion from the donor cell's mtDNA (complex nucleoid organization) introduced by SCNT was not detected by the autophagic cell mechanisms of the embryo, and allowed the persistence of this artificial heteroplasmy during the different stages of development in the bovine clones. In the natural heteroplasmy produced by the mtDNA common deletion, this huge deletion in the oocyte's mtDNA (simpler nucleoid organization) probably was detected by the autophagic cell mechanisms of the embryo, and was eliminated to guarantee the beginning of embryo's life in conditions of homoplasmy, similarly to what is described by Bogenhagen.(BOGENHAGEN 2010).

Finally, the results of both studies were quite different because the segregation of mutated mtDNA is a complex phenomenon that depends of many factors: if the mtDNA mutation analyzed is introduced by an artificial technique (SCNT) or is produced by a natural process (aging); if the mtDNA mutation at early embryonic stage is introduced in a somatic cell (complex nucleoid organization) or is produced in a germinal cell (simpler nucleoid organization); if the mitochondria from donor and recipient cells are fused after the process of electrofusion in SCNT experiments; also depends on the size (bp) of mutation, the type of the analyzed mutation (point mutation, insertion or deletion) and the stage of development of the analyzed cells (germinal, embryonic or somatic cells). Probably, there are more factors involved in the mtDNA segregation in these experiments that we did not observe or we did not discuss. All the interpretations of different results in

mitochondrial DNA segregation experiments must be explained at cellular and molecular levels to better understand this phenomenon.

Further experiments must be performed, preferably in the same species, to elucidate the meaning of these differences in the segregation patterns of the mtDNA produced by artificial and natural heteroplasmy, and the mechanisms involved to preserve, increase or eliminate the mutated mtDNA since the embryonic stage until adulthood life.

# General conclusions

5.1. - The heteroplasmy produced by the introduction of foreign mtDNA from somatic cells to enucleated oocytes was very stable from oocyte reconstruction until adult life. This neutral segregation of mtDNA could be explained by fusion of the two sorts of mitochondria at the moment of electrofusion, and the consequent sharing of the two mtDNAs in the same nucleoid population.

5.2. - Higher levels of heteroplasmy were found in the tissues of one bovine SCNT clone. This higher percentage of mtDNA from the donor cell could be explained by successful fusion of the cells and unsuccessful fusion of the mitochondria at the time of electrofusion, leaving the two mtDNA in different nucleoid populations with stochastic segregation of the mtDNA.

5.3. - Female mouse fertility decreased with aging but this decrease was not related to the integrity of mtDNA from oocytes.

5.4. - Oocytes were not affected by the process of aging like somatic tissues. Granulosa cells are exceptional among somatic cells not affected by the process of aging.

5.5. - In vitro culture did not affect the quality of oocytes and embryos for most of the mitochondrial parameters analyzed.

5.6. - There was a huge increase in the percentage of the mitochondrial DNA common deletion at the resumption of the meiosis. At the beginning of the embryonic period this huge percentage disappeared by unknown mechanisms. We suggest two possible mechanisms for the removal of mutated mtDNA: the elimination of oocytes harbouring mtDNA common deletion by apoptosis, or elimination of damaged mitochondria (harbouring mutated mtDNA) by a process of autophagy.

5.7. - We suggest that in germinal cells, the mtDNA common deletion is more related with the energy pathway at different stages of development than with aging.

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